

PHARMACOKINETICS OF MONTELUKAST AND ZAFIRLUKAST AS AFFECTED BY CYP INHIBITORS

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ACADEMIC DISSERTATION

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To Julie

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ABBREVIATIONS

| | |
|--------------------|---|
| AA | arachidonic acid |
| ADME | absorption, distribution, metabolism and excretion |
| ADR | adverse drug reaction |
| Ah | aryl hydrocarbon |
| ANOVA | analysis of variance |
| ASA | acetylsalicylic acid |
| ATP | adenosine triphosphate |
| AU | arbitrary unit |
| AUC | area under the plasma drug concentration-time curve |
| BBB | blood-brain barrier |
| BCE | before the Common Era |
| BCRP | breast cancer resistance protein |
| BLT ₁ | leukotriene B ₄ receptor 1 |
| BLT ₂ | leukotriene B ₄ receptor 2 |
| BMI | body mass index |
| B-PVK | basic blood count |
| CAR | constitutive androstane receptor |
| CL | clearance |
| C _{max} | peak plasma concentration |
| COX | cyclo-oxygenase |
| CSF | cerebrospinal fluid |
| CV | coefficient of variation |
| CYP | cytochrome P450 |
| CysLT ₁ | cysteinyl leukotriene receptor 1 |
| CysLT ₂ | cysteinyl leukotriene receptor 2 |
| DNA | deoxyribonucleic acid |
| DPP | dipeptidylpeptidase |
| EDTA | ethylenediaminetetraacetic acid |
| EETs | epoxyeicosatrienoic acids |
| EH | epoxide hydrolase |
| EM | extensive metaboliser |
| EMA | European Medicines Agency |
| EU | European Union |
| FDA | Food and Drug Administration |
| FEV ₁ | forced expiratory flow in one second |
| FLAP | 5-lipoxygenase activating protein |
| GST | glutathione S-transferase |
| Hb | haemoglobin |
| HDL | high density lipoprotein |
| HLMs | human liver microsomes |
| HMG-CoA | hydroxymethylglutaryl coenzyme A |
| HPLC | high performance liquid chromatography |
| IC ₅₀ | inhibitor concentration producing 50% inhibition |
| IgE | immunoglobulin E |
| IL | interleukin |
| IM | intermediate metaboliser |
| INR | international normalised ratio |

| | |
|------------------|---|
| IS | internal standard |
| k_e | elimination rate constant |
| K_i | inhibitor concentration supporting half of the maximal inhibition |
| K_m | Michaelis-Menten kinetic constant |
| LABA | long-acting β_2 -agonist |
| LC/MS/MS | liquid chromatography-tandem mass spectrometry |
| LDL | low density lipoprotein |
| LLQ | lower limit of quantification |
| 5-LO | 5-lipoxygenase |
| LT | leukotriene |
| LTRA | leukotriene receptor antagonist |
| M | metabolite |
| MDCKII | Madin-Darby canine kidney II |
| MDR | multidrug resistance |
| MgOH | magnesiumhydroxide |
| M-M | Michaelis-Menten |
| mRNA | messenger ribonucleic acid |
| MRP | multidrug resistance-associated protein |
| MT | methyltransferase |
| NaCHO_3 | sodiumbicarbonate |
| NADH | nicotinamide adenine dinucleotide, reduced |
| NADPH | nicotinamide adenine, dinucleotide phosphate, reduced |
| NAT | <i>N</i> -acetyltransferase |
| NO | nitric oxide |
| NSAID | nonsteroidal anti-inflammatory drug |
| OAT | organic anion transporter |
| OATP | organic anion-transporting polypeptide |
| OCT | organic cation transporter |
| P-AFOS | plasma alkaline phosphatase |
| P-ALAT | plasma alanine aminotransferase |
| PBPK | physiologically based pharmacokinetic |
| PCR | polymerase chain reaction |
| PEF | peak expiratory flow |
| PG | prostaglandin |
| P-gp | P-glycoprotein |
| P-GT | plasma gamma-glutaryl transferase |
| P-K | plasma potassium |
| P-Krea | plasma creatinine |
| PLA_2 | phospholipase A_2 |
| PM | poor metaboliser |
| P-Na | plasma sodium |
| PPAR | peroxisome proliferator-activated receptor |
| PPI | proton pump inhibitor |
| PXR | pregnane X receptor |
| RA | rheumatoid arthritis |
| rh | recombinant human |
| SD | standard deviation |
| SEM | standard error of the mean |
| S-hCG | serum human chorionic gonadotropin |
| SLC | solute carrier family |

| | |
|-----------|--|
| SLCO | solute carrier organic anion transporter family |
| s/n | signal-to-noise ratio |
| SNP | single-nucleotide polymorphism |
| SRS-A | slow-reacting substance of anaphylaxis |
| SSRI | selective serotonin reuptake inhibitor |
| ST | sulfotransferases |
| $t_{1/2}$ | elimination half-life |
| TDM | therapeutic drug monitoring |
| TdP | Torsades de Pointes |
| t_{max} | time to reach C_{max} |
| TNF | tumor necrosis factor |
| UDP | uridine 5'-diphosphate |
| UGT | uridine 5'-diphosphate (UDP) glucuronosyltransferase |
| UM | ultra-rapid metaboliser |
| US | United States |
| VLDL | very low density lipoprotein |
| V_{max} | maximum velocity |

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text as studies I–IV:

- I Karonen T, Filppula A, Laitila J, Niemi M, Neuvonen PJ, Backman JT.
Gemfibrozil markedly increases the plasma concentrations of montelukast: a previously unrecognized role for CYP2C8 in the metabolism of montelukast.
Clin Pharmacol Ther. 2010 Aug;88(2):223–230.
- II Karonen T, Neuvonen PJ, Backman JT.
CYP2C8 but not CYP3A4 is important in the pharmacokinetics of montelukast.
Br J Clin Pharmacol. 2012 Feb;73(2):257–267.
- III Karonen T, Neuvonen PJ, Backman JT.
The CYP2C8 inhibitor gemfibrozil does not affect the pharmacokinetics of zafirlukast.
Eur J Clin Pharmacol 2011 Feb;67(2):151–155.
- IV Karonen T, Laitila J, Niemi M, Neuvonen PJ, Backman JT.
Fluconazole but not the CYP3A4 inhibitor itraconazole increases plasma concentrations of zafirlukast.
Eur J Clin Pharmacol 2012 May;68(5):681–688.

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ABSTRACT

Montelukast and zafirlukast are leukotriene receptor antagonists that have been available for the treatment of asthma and allergic rhinitis for over 15 years. At the time of their marketing authorisations the knowledge of their metabolic pathways was largely based on *in vitro* studies. The main enzymes catalysing the biotransformation of montelukast *in vitro* were cytochrome P450 (CYP) 2C9 and CYP3A4. Relatively high montelukast concentrations were used in these studies, and CYP2C8 was not included. The enzymes participating in the *in vitro* metabolism of zafirlukast were also CYP2C9 and CYP3A4.

Montelukast and zafirlukast are both potent inhibitors of CYP2C8 *in vitro*, but neither of them has shown inhibitory effect on CYP2C8 *in vivo*. Montelukast has also been shown to fit well in the active site cavity of CYP2C8 in a crystallography study. These observations led to examine the role of CYP2C8 and CYP3A4 in the metabolism of montelukast in humans, as well as the role of CYP2C8, CYP2C9 and CYP3A4 in the metabolism of zafirlukast in humans.

This work was carried out as four clinical drug interaction studies in healthy volunteers with a randomised, placebo-controlled cross-over design. The inhibitor drugs used were gemfibrozil for CYP2C8, fluconazole for CYP2C9 and CYP3A4 and itraconazole for CYP3A4. The inhibition of CYP2C8 resulted in over fourfold increase of the area under the plasma drug concentration-time curve (AUC) of montelukast, and almost a cessation of the formation of its major metabolite M4. Inhibition of CYP3A4 had no effect on the total elimination of montelukast, and only decreased the formation of a minor metabolite M5. The pharmacokinetics of zafirlukast were only affected by inhibition of CYP2C9, which resulted in a 1.6-fold increase of the AUC of zafirlukast. Inhibition of CYP2C8 and CYP3A4 had no effect on the pharmacokinetics of zafirlukast.

The results of this work elucidated that CYP2C8 accounts for about 80% of the metabolism of montelukast in humans, while CYP3A4 has no significant role in it. Concomitant use of CYP2C8 inhibitors with montelukast may increase the risk of concentration dependent adverse drug reactions (ADRs). Further the work showed that the main enzyme in the metabolism of zafirlukast in humans is CYP2C9. Thus, although zafirlukast has a wide safety margin, concomitant use of zafirlukast with CYP2C9 inhibitors may increase the risk of concentration dependent ADRs. Moreover, with regard to drug interaction studies, montelukast is a promising candidate as a sensitive and relatively safe *in vivo* probe drug for CYP2C8.

Both montelukast and zafirlukast were developed and granted marketing authorisations before the publication of the regulatory guidelines for drug interaction studies during drug development, and since then the understanding of the importance of CYP enzymes, especially CYP2C8, in drug metabolism has increased markedly. These results highlight the relevance of drug interaction studies and the regulatory guidelines related to them. Especially drugs that have been developed before the existence of these guidelines may be deficiently characterised with regard to their metabolism, leaving the possibility of unrecognised CYP-mediated interactions.

INTRODUCTION

Drug interactions are potentially hazardous and may result in serious adverse drug reactions, even fatalities. Further, in many cases serious drug interactions have led to the withdrawal of the drug from the market (Huang *et al.* 2008). Therefore the interaction potential of a new drug candidate needs be carefully examined during drug development. The drug interactions studies are usually carried out in healthy volunteers, and the probe substrate, inhibitor and inducer drugs should be as safe as possible. For example, due to its blood glucose lowering effect, the most used cytochrome P450 (CYP) 2C8 probe substrate, repaglinide, requires blood glucose level monitoring and possibly rescue interventions during drug interactions studies.

Montelukast and zafirlukast are leukotriene receptor antagonists used in the treatment of asthma and allergic rhinitis, which are common morbidities in developed countries, affecting about 7% and 23% of the population, respectively. Montelukast and zafirlukast act by decreasing the inflammatory responses, such as bronchoconstriction, mediated by leukotrienes. The marketing authorisations of montelukast and zafirlukast were granted in 1996–1997, and thus these drugs were developed during an era when the importance of drug interactions and the central role of CYP enzymes in drug interactions were recognised, but no formal guidance for the industry existed yet. At the time of the marketing authorisations of montelukast and zafirlukast the enzymes involved in their metabolism in humans were not known, as the knowledge of their biotransformation was mainly based on *in vitro* studies. The main biotransformation reactions of montelukast *in vitro* were catalysed by CYP2C9 and CYP3A4, when studied in high concentrations, compared to those reached in clinical practice (Chiba *et al.* 1997). These enzymes also participated in the *in vitro* metabolism of zafirlukast (Kassahun *et al.* 2005; Accolate Label 2011).

The interaction of gemfibrozil and cerivastatin resulting in even fatal cases of rhabdomyolysis brought attention to the importance of CYP2C8 in drug metabolism (Huang *et al.* 2008). This interaction was subsequently characterised to occur via mechanism-based inhibition of CYP2C8 by the glucuronide metabolite of gemfibrozil (Chang *et al.* 2004; Neuvonen *et al.* 2006; Ogilvie *et al.* 2006). Subsequently CYP2C8 was further characterised in structure and substrate binding, and screened widely for *in vitro* inhibitor molecules. Montelukast and zafirlukast were among the most potent CYP2C8 inhibitors from 209 drugs screened, montelukast being a very selective, competitive inhibitor of CYP2C8 (Walsky *et al.* 2005a; Walsky *et al.* 2005b). Montelukast also fitted well to the active site cavity of CYP2C8 in a crystallography modelling (Schoch *et al.* 2008). Montelukast and zafirlukast did, however, not affect the pharmacokinetics of CYP2C8 substrates, such as repaglinide and pioglitazone, *in vivo* (Jaakkola *et al.* 2006a; Kajosaari *et al.* 2006).

These findings together initiated an interest to study the role of CYP2C8 in the metabolism of montelukast and zafirlukast themselves. The inhibitory effect of gemfibrozil and gemfibrozil 1-O- β glucuronide on the *in vitro* metabolism of montelukast was studied in human liver microsomes (HLM). In addition the effect of gemfibrozil on the pharmacokinetics of montelukast and zafirlukast was studied in healthy volunteers. The possible roles of CYP3A4 in the biotransformation of montelukast and CYP2C9 and CYP3A4 in the biotransformation of zafirlukast were also of interest. The effect of itraconazole and its combination with gemfibrozil on the pharmacokinetics of montelukast, as well as the effect of fluconazole and itraconazole on the pharmacokinetics of zafirlukast was studied in healthy volunteers.

REVIEW OF THE LITERATURE

1 Asthma and allergic rhinitis

Atopy is an abnormal tendency to develop specific immunoglobulin E (IgE) in response to harmless and ubiquitous allergens. Atopic diseases include asthma, allergic rhinoconjunctivitis, atopic dermatitis and food allergies, and tend to run in families (Greiner *et al.* 2011). Atopy is a significant comorbidity of asthma, as about 10% of atopic individuals develop asthma, while about 50% of asthma patients are also atopic.

Asthma is a heterogenic inflammatory disease characterised by airway inflammation, bronchial hyper-reactivity and reversible airway obstruction, manifesting as e.g. dyspnoea, wheezing, coughing and chest tightness. The airway obstruction may also culminate as an asthma attack, which is a potentially fatal condition. Allergic asthma is the most common form of asthma, and it is characterised by the production of allergen specific IgE, which then with inhaled allergens may trigger acute exacerbation of the symptoms (Bhakta *et al.* 2011; Mullane 2011).

Asthma affects over 300 million people in developed countries, with a prevalence of about 7% among adults in the United States (US) (Weiss *et al.* 2006; Bhakta *et al.* 2011). Asthma is the most common chronic disease in children in developed countries, and globally about 6% of 6–14 year old children have had asthma and have current wheeze (Pearce *et al.* 2007; Bhakta *et al.* 2011). The prevalence of asthma has increased considerably within the past 20–30 years, although with indications of decreases in prevalence in English speaking countries and Western Europe (Pearce *et al.* 2007).

Although asthma patients have similar symptoms and physiologic abnormalities of the airways, asthma is actually a spectrum of diseases. It is very heterogenic with respect to e.g. genetic susceptibility, age of onset, sensitivity to the triggers causing the airway obstruction, the frequency of exacerbations, the severity of the airway obstruction and symptoms, and the degree of reversibility and responsiveness to treatments (Bhakta *et al.* 2011; Mullane 2011; Upham *et al.* 2011).

Allergic rhinitis is the most common form of non-infectious rhinitis and a frequent comorbidity of asthma. Allergic rhinitis is also much more common than asthma, and worldwide it is estimated to affect about 500 million people, and the prevalence in Europe is about 23% (Bauchau *et al.* 2004; Bousquet *et al.* 2008). About 80% of asthma patients also have allergic rhinitis, whereas 10–40% of patients with allergic rhinitis have asthma. Similarly as for asthma, the prevalence of allergic rhinitis has increased during the past few decades (Bousquet *et al.* 2001; Bousquet *et al.* 2008).

1.1 Pathogenesis of asthma and allergic rhinitis

The pathogenesis of asthma is multifactorial, including both genetic and environmental factors. There are descriptions of the clinical picture and postulations about the pathophysiology of asthma dating already before the Common Era (BCE). The understanding of the underlying

mechanisms has expanded vastly during the past 100 years, and will presumably continue to do so in the future along the advances in genetics, genomics and proteomics (Walter *et al.* 2005). These advances affect also the understanding of the treatment of asthma. Over 40 human genes have been identified to associate with asthma (Rogers *et al.* 2009), and there have been estimates that genetics contribute to as much as 60–80% of the interindividual variability of treatment response (Weiss *et al.* 2006).

Numerous cells and mediators have been identified to play a role in the pathogenesis of asthma, but in a simplified way it can be described as follows (Rang *et al.* 2007b):

- exposure of genetically disposed individuals to allergen
- activation of Th2 lymphocytes and cytokine generation
- production of and release of IgE
- expression of IgE receptors on mast cells and eosinophils
- generation and release of other important mediators, such as leukotriene B₄ (LTB₄), cysteinyl leukotrienes (LTC₄ and LTD₄), interleukins (IL-4, -5, -13) and tissue damaging eosinophil proteins

Allergic rhinitis has only been described since the early 19th century, at which time it was still very uncommon, and was defined the first time in 1929 (Bousquet *et al.* 2001; Bousquet *et al.* 2008). Allergic rhinitis is an inflammatory disorder of nasal mucosa, manifesting as pruritus, sneezing, rhinorrhoea and nasal congestion. It is mediated by similar hypersensitivity responses to environmental allergens as allergic asthma, and the inflammatory response is characterised by IgE synthesis, IgE-dependent mast cell activation and infiltration of T lymphocytes and eosinophils to the nasal mucosa (Durham 1998; Bousquet *et al.* 2001; Bousquet *et al.* 2008). The diagnosis is often simple and based on the typical symptoms. However, allergic rhinitis may still be under-diagnosed, as many patients do not consider their symptoms of rhinitis as a disease impairing their social life, school and work, which allergic rhinitis nevertheless has been shown to do (Greiner *et al.* 2011).

1.2 Drugs used in the treatment of asthma and allergic rhinitis

The treatment of asthma is currently targeted at the inflammation of the airways and bronchoconstriction, and consists of two main categories, anti-inflammatory agents (also so-called controller drugs) and bronchodilators (reliever drugs). The choice of treatment is complex and depends on the severity of the disease and the treatment response of the patient. Several treatment guidelines exist (British Guideline on the Management of Asthma 2008).

The main bronchodilator drugs are short-acting β_2 -agonists, such as salbutamol and terbutaline. They very rarely suffice as the only treatment for asthma, but may do so for very occasional mild symptoms, alone or with short courses of inhaled corticosteroids. An increasing need for the use of short-acting β_2 -agonists indicates poor control of the disease and need for anti-inflammatory treatment (British Guideline on the Management of Asthma 2008).

Long-acting β_2 -agonists (LABAs), such as salmeterol and formoterol, are also used in the treatment of asthma, in combination with inhaled corticosteroids. Their use has been associated with development of tolerance and decreased efficacy of simultaneously used short-acting β_2 -agonists (van der Woude *et al.* 2001; Lee *et al.* 2004). Other bronchodilator

drugs include xanthine drugs (e.g. theophylline), muscarinic receptor antagonists (e.g. ipratropium) and cysteinyl leukotriene receptor antagonists (LTRAs, e.g. montelukast).

The anti-inflammatory agents include glucocorticoids, cromoglicate and nedocromil, as well as anti-IgE-treatment (omalizumab). LTRAs are also thought to act to some extent on the inflammatory component of asthma. Inhaled corticosteroids like fluticasone, budesonide and beclomethasone are the basis of the treatment of the inflammation of the airways, and have been the golden standard of the treatment of asthma for decades. Their early use may prevent a worsening of the lung function (Lange *et al.* 1998), and reduce the need for hospital treatment (Blais *et al.* 1998). They can be used periodically in mild forms of asthma, but are usually needed as continuous treatment in the more severe forms of the disease.

The adverse effects of inhaled corticosteroids are also well known, and increase with the increase of doses, examples including bruising and thinning of the skin (Leone *et al.* 2003). Inhaled doses of over 800 µg daily of beclomethasone or budesonide or over 400 µg daily of fluticasone may affect the function of the adrenal cortex and the metabolism of both children and adult patients (Turpeinen *et al.* 1991; Sorva *et al.* 1992). The short-term growth rate may decrease in children during inhaled corticosteroid treatment, and may even persist into adulthood (Leone *et al.* 2003; Kelly *et al.* 2012). Some patients may thus benefit from other treatment options for asthma that may allow the reduction of the dose of inhaled corticosteroids.

The future goal for asthma treatment could be achievement of long-term remission, as in other chronic inflammatory diseases like e.g. rheumatoid arthritis. Spontaneous asthma remissions have been observed, most frequently during adolescence and early adulthood. A better understanding of the mechanisms leading to spontaneous remission might provide insights into how to achieve remission of asthma with treatment. (Upham *et al.* 2011)

The concomitant treatment of asthma with inhaled or orally administered corticosteroids, short- or long-acting β_2 -agonists or theophylline with LTRAs does not necessitate dose adjustments of any of these drugs (Accolate Label 2011; Singulair Label 2011). The majority of an inhaled β_2 -agonist dose is swallowed, and roughly 10–30% reaches the lung. The swallowed fraction of e.g. salbutamol undergoes extensive intestinal and hepatic first-pass conjugation, whereas, similarly as for inhaled corticosteroids, no first-pass metabolism occurs in the lung (Lipworth 1996).

Most of an inhaled corticosteroid dose is also swallowed, and a similar fraction as for inhaled β_2 -agonists reaches the lung. Fluticasone and budesonide undergo a high degree (up to 90%) of first-pass metabolism of the swallowed dose, and beclomethasone in a lower degree (Lipworth 1996). The metabolism of both inhaled and orally administered corticosteroids is mainly mediated by CYP3A4. Itraconazole has increased the plasma concentrations of inhaled budesonide in healthy volunteers (Raaska *et al.* 2002). In keeping with this, there are also several case reports of iatrogenic adrenal suppression and Cushing's syndrome in patients receiving inhaled corticosteroids concomitantly to a CYP3A4 inhibitor (Foisly *et al.* 2008; Valin *et al.* 2009).

The xanthine drug theophylline is mainly metabolised by CYP1A2, which is inhibited by e.g. alcohol, and induced by e.g. cigarette smoke (Zevin *et al.* 1999; Carrillo *et al.* 2000). Concomitant intake of theophylline and coffee has resulted in decreased elimination of both these compounds, caffeine also being a substrate of CYP1A2 (Sato *et al.* 1993). In smokers

teophylline clearance is significantly increased compared to non-smokers (Zevin 1999). Teophylline is thus susceptible to interactions with drugs or other factors affecting the activity of CYP1A2.

The pharmacological treatment of allergic rhinitis is well established, and several guidelines exist (Bousquet *et al.* 2010). Intranasal corticosteroids are most effective, have effect on all the symptoms of allergic rhinitis and are the only pharmacological treatments that also restrain the development of nasal polyps. Oral or intranasal antihistamines and montelukast are also recommended. Topical cromones have only a modest effect, and intranasal decongestants may be used for short periods in case of severe nasal obstruction. (Bousquet *et al.* 2008)

2 Leukotrienes

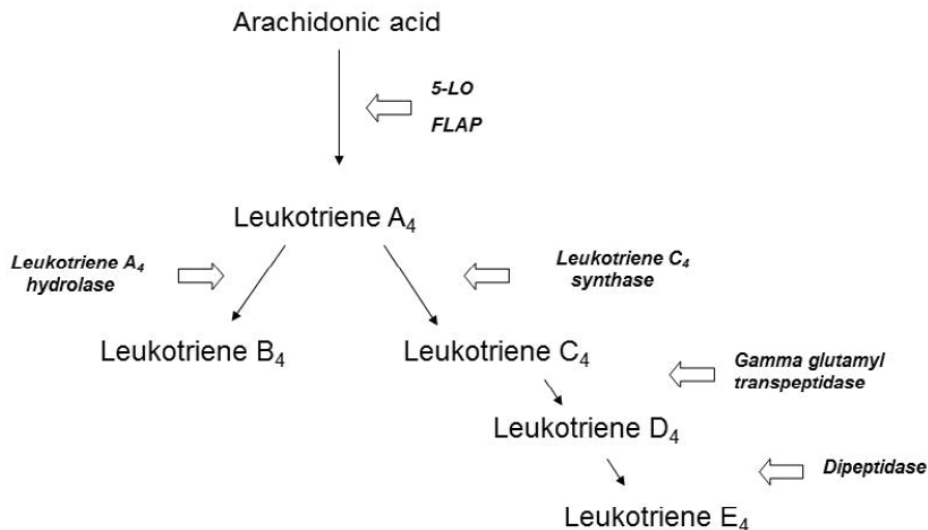
Leukotrienes were formerly known as the slow-reacting substance of anaphylaxis (SRS-A), which was identified along the discovery of antihistamines in the 1950s as the non-histamine mediator to cause smooth muscle constriction. In the 1980s SRS-A was elucidated to be composed of cysteinyl leukotriene C₄ (LTC₄) and its active metabolites LTD₄ and LTE₄, which participate in the pathophysiology of asthma. (Lewis *et al.* 1980; Morris *et al.* 1980; Samuelsson *et al.* 1980; Samuelsson 1983; Lane 1998; Boyce 2008)

Leukotriene receptor antagonists (LTRAs) have been available for the treatment of asthma and allergic rhinitis for 15 years, and are all administered orally. Montelukast and zafirlukast are available worldwide, and pranlukast only in Japan. LTRAs are a hybrid of anti-inflammatory and bronchodilator treatment. They antagonise the pro-inflammatory activities of leukotrienes and the leukotriene induced smooth muscle bronchoconstriction, without altering the smooth muscle response to β_2 -agonists (Lipworth 1999).

2.1 The 5-lipoxygenase pathway

Leukotrienes are produced on demand from arachidonic acid (AA) upon cellular activation, when AA is released from the membrane phospholipids by phospholipase A₂ (PLA₂). The stimuli leading to the cellular activation and release of AA are multiple, including cytokines, antigen-antibody reactions on mast cells or general cell damage. The released AA can be further metabolised by a variety of pathways, leading to the production of prostaglandins and thromboxanes by the cyclo-oxygenase (COX) pathway, or leukotrienes resulting from the 5-lipoxygenase (5-LO) pathway (Figure 2.1.1). These three groups of eicosanoids are needed both in the control of many physiological processes, as well as in being among the most important mediators and modulators of inflammatory reaction. The pathway activated depends on the specific cell type and the activating stimuli (Lane 1998; Wenzel 2003; Boyce 2008). AA can also be further metabolised by a third pathway, the CYP epoxygenase pathway, which results in the epoxidation of AA to epoxyeicosatrienoic acids (EETs) catalysed by CYP2J and CYP2C enzymes. This pathway is an important regulator of cardiovascular inflammation and vascular tone (Deng *et al.* 2010).

Figure 2.1.1 The 5-LO pathway



The 5-LO pathway is a sequential process and regulated on multiple levels. The cellular synthesis of leukotrienes is limited to cells expressing both 5-LO and 5-LO activating protein (FLAP), and cells of myeloid lineage are the major source for leukotriene production. The 5-LO first catalyses the formation of the unstable leukotriene A₄ (LTA₄). Intracellular predominance of either LTA₄ hydrolase or LTC₄ synthase (glutathione S-transferase) then determines the ability of the cell to preferentially produce either LTB₄ or the cysteinyl leukotrienes LTC₄, LTD₄ and LTE₄ (Figure 2.1.1). Phagocytic cells (neutrophils, monocytes, macrophages) produce mainly LTB₄, whereas cysteinyl leukotrienes are generated mainly by eosinophils, mast cells and basophils. (Lane 1998; Boyce 2008)

The cysteinyl leukotrienes are potent bronchoconstrictors, LTC₄ and LTD₄ being quite equipotent and approximately 2000 times more potent than histamine or methacholine, while LTE₄ is 30–100 times more potent than histamine (Brink *et al.* 2003; Wenzel 2003). The cysteinyl leukotrienes are also potent stimulators of mucus secretion, and convey smooth muscle contraction and contribute to extravascular leakage. They are also chemoattractant to and activators of neutrophils. The possible role of cysteinyl leukotrienes in the modulation of structural changes, i.e. airway remodelling seen in asthma remains to be further clarified (Wenzel 2003; Durrani *et al.* 2011).

2.2 Leukotriene receptors

The leukotrienes convey their effects via the leukotriene receptors on cell surface, which are G-protein coupled receptors (Duroudier *et al.* 2009). Two LTB₄ receptors are known; the leukotriene B₄ receptor 1 (BLT₁) that is present on mononuclear cells and macrophages, and

leukotriene B₄ receptor 2 (BLT₂) present on structural cells and lymphocytes (Yokomizo *et al.* 1997; Kamohara *et al.* 2000; Yokomizo *et al.* 2000; Wenzel 2003).

For the cysteinyl leukotrienes at least two cysteinyl leukotriene receptors (CysLT₁ and CysLT₂) have been identified (Labat *et al.* 1992; Tudhope *et al.* 1994; Capra 2004), and confirmed as two distinct receptors when their encoding genes were cloned, the CysLT₁ reported first (Lynch *et al.* 1999; Sarau *et al.* 1999; Heise *et al.* 2000; Nothacker *et al.* 2000; Takasaki *et al.* 2000). CysLT₁ messenger ribonucleic acid (mRNA) was found to be expressed in several tissues, including e.g. lung, bronchus and nasal mucosa, peripheral leukocytes, heart, brain, kidney and gastrointestinal system (Brink *et al.* 2003; Capra 2004). *In vitro* the affinities of the cysteinyl leukotrienes for the CysLT₁ where in the following order: LTD₄>>LTE₄= LTC₄>>LTB₄, with 3000 fold difference between the inhibitor concentration producing 50% inhibition (IC₅₀) of LTD₄ and LTB₄ (Lynch *et al.* 1999). In functional studies LTE₄ has appeared to be less potent in activating the cysteinyl leukotriene receptors than LTC₄ and LTD₄ (Brink *et al.* 2003).

The CysLT₂ mRNA has been found in the human heart, adrenals, peripheral leukocytes, placenta, spleen and lymph nodes, and to lesser degree in the brain (Heise *et al.* 2000; Takasaki *et al.* 2000). *In situ* hybridisation studies in human lung have shown strong expression of CysLT₂ in the interstitial macrophages and weak expression in the smooth muscle cells (Heise *et al.* 2000; Brink *et al.* 2003).

In conclusion, CysLT₁ mediates the constriction of the bronchi and CysLT₂ the constriction of vascular smooth muscles. Most of the biological effects of cysteinyl leukotrienes, including bronchospasm, plasma exudation, vasoconstriction, mucus secretion and eosinophil recruitment are mediated via the CysLT₁ receptor subtype. The CysLT₁ is also the target of LTRAs, with montelukast having the highest binding affinity, whereas the CysLT₂ is not significantly antagonised by montelukast, zafirlukast or pranlukast (Lynch *et al.* 1999; Heise *et al.* 2000; Brink *et al.* 2003).

3 Montelukast

Montelukast is the most widely used LTRA, and was among the top 10 drugs in the US by the amount of prescriptions dispensed in 2009. It was introduced to the market in 1997 under trade name Singulair, and is currently also available as several generic preparations. In the European Union (EU) and the US montelukast is indicated for the prophylaxis and chronic treatment of asthma, the prevention of exercise induced bronchoconstriction, and for the relief of symptoms of allergic rhinitis in adult and pediatric patients. Montelukast is a selective, potent and competitive antagonist of the CysLT₁: it antagonised the binding of LTD₄ to CysLT₁ with an IC₅₀ ranging from 1.2 to 2.3 nM, which was of similar potency than zafirlukast, and more potent than pranlukast (Lynch *et al.* 1999; Sarau *et al.* 1999). The characteristics and pharmacokinetic properties of montelukast are summarised in Table 3.1.

Table 3.1. Characteristics of montelukast

| Montelukast | |
|--|---|
| Chemical formula and name | C ₃₅ H ₃₅ ClNNaO ₃ S [<i>R</i> -(<i>E</i>)]-1-[[[1-[3-[2-(7-chloro-2-quinolinyl)ethenyl]phenyl]-3-[2-(1-hydroxy-1-methylethyl)phenyl]propyl]thio]methyl]cyclopropaneacetic acid, monosodium salt |
| Molecular weight | 608.18 |
| <i>In vitro</i> metabolism | Metabolite M6 formation: CYP2C8>>CYP2C9 M5 formation: CYP3A4 |
| <i>In vitro</i> inhibitory effect (HLMs) | CYP2C8: IC ₅₀ 0.02 µM CYP2C9: IC ₅₀ 1.2 µM CYP3A: IC ₅₀ 1.2–7.9 µM Other CYP enzymes: IC ₅₀ > 10 µM |
| Antagonism of LTD ₄ binding to CysLT ₁ | IC ₅₀ : 1.2–2.3 nM |
| Daily dose | 10mg (>15 y), 5 mg (6–14 y), 4 mg (2–5 y) once in the evening |
| Oral bioavailability | 63–73% |
| Plasma protein binding | >99% |
| Volume of distribution | 8–11 l |
| t _{max} | 3 h (10 mg tablet), 2–2.5 h (5 and 4 mg tablet, 4 mg oral granule) |
| t _{1/2} | 2.7–5.5 h |
| Elimination | Extensive metabolism: CYP2C8, 2C9 and 3A4 (based on <i>in vitro</i> studies) Excretion almost exclusively via bile (metabolites and unchanged), 0.2% in urine |
| Transporters | Substrate of OATP2B1 Inhibitor of MRP2 <i>in vitro</i> |

(Chiba *et al.* 1997; Lynch *et al.* 1999; Sarau *et al.* 1999; Walsky *et al.* 2005b; Mougey *et al.* 2009; Roy *et al.* 2009b; Filppula *et al.* 2011; Singulair Label 2012)

| | |
|--------------------|--|
| HLMs | human liver microsomes |
| IC ₅₀ | inhibitor concentration producing 50% inhibition |
| LT | leukotriene |
| CysLT ₁ | cysteinyl leukotriene receptor 1 |
| t _{max} | time to reach peak plasma concentration |
| t _{1/2} | elimination half-life |
| OATP | organic anion-transporting polypeptide |
| MRP | multidrug resistance-associated protein |

3.1 Metabolism of montelukast

Montelukast is extensively metabolised in the liver to one major and several minor metabolites, which are excreted in the bile. After an oral dose of radiolabelled montelukast, 86% of the radioactivity was detected in a five day faecal collection, and less than 0.2% in urine, indicating almost exclusive biliary excretion for montelukast and its metabolites (Singulair Label 2012).

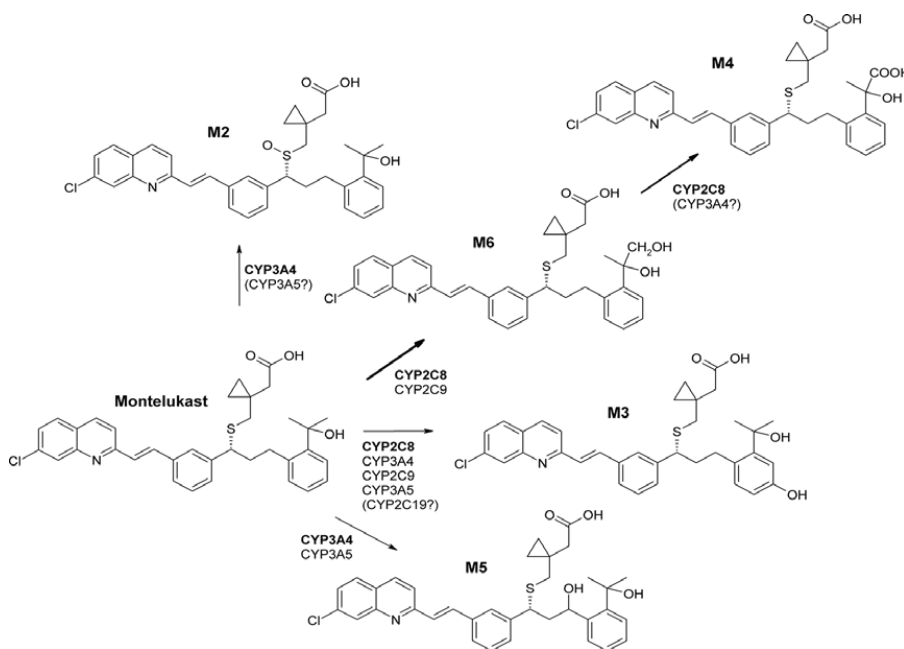
The metabolites have been identified as following: the major metabolite M4 is a dicarboxylic acid, resulting from further oxidation of a primary metabolite, 36-hydroxy of a methyl alcohol,

M6. The minor metabolites are acyl glucuronide M1, sulfoxide M2, 25-hydroxy (a phenol) M3 and 21-hydroxy diastereomers of a benzylic alcohol M5a and M5b. (Balani *et al.* 1997; Chiba *et al.* 1997)

According to the Singulair prescribing information, montelukast is metabolised mainly by CYP3A4 and CYP2C9 (Singulair Label 2012). This is based on *in vitro* studies in human liver microsomes (HLMs) with high montelukast concentrations (100–500 $\mu\text{mol/l}$), and the role of CYP2C8 was not studied at that time. The 21-hydroxylation to form M5 was catalysed by CYP3A4 and the formation of M6 via 36-hydroxylation by CYP2C9 (Chiba *et al.* 1997).

Recently the *in vitro* metabolism of montelukast has been studied with lower montelukast concentrations (1–10 μM in HLMs and 0.1 and 0.02 μM in recombinant human (rh) CYP isoforms), and the importance of CYP2C8 in its metabolism was shown (Filppula *et al.* 2011). In HLMs the CYP2C8 inhibitors gemfibrozil 1-O- β glucuronide and trimethoprim inhibited the depletion of montelukast and formation of M6 from montelukast more efficiently than CYP2C9 inhibitor sulfaphenazole. RhCYP2C8 catalysed the depletion of montelukast, and formation of M6 with sixfold higher intrinsic clearance than CYP2C9, and other P450 isoforms produced no M6. In addition it was verified that M5a/b is mainly formed via CYP3A4. Based on the depletion of 0.02 μM montelukast the authors estimated that CYP2C8 would account for 72% of the *in vivo* oxidative metabolism of montelukast, CYP3A4 for 16% and CYP2C9 for 12% (Filppula *et al.* 2011). These results have been verified in a further *in vitro* study where CYP2C8 and CYP3A4 were the major CYP enzymes involved in montelukast metabolism (VandenBrink *et al.* 2011). Figure 3.1.1 presents the formation of montelukast metabolites and the catalysing enzymes, as based on these studies (Chiba *et al.* 1997; Filppula *et al.* 2011).

Figure 3.1.1 Metabolism of montelukast *in vitro* (Filppula *et al.* 2011)



In a crystallography modelling montelukast has been found to fit well in the active site cavity of CYP2C8, which is an extended, three-branch cavity and able to oxidise substrates with high molecular weight (Schoch *et al.* 2004; Schoch *et al.* 2008). Montelukast was positioned in the active cavity with its benzyl ring near the heme iron of CYP2C8, and as described above, the major metabolite of montelukast, M4 and its precursor, M6, result from the oxidation of the benzyl ring of montelukast. This finding indicates a possible role for CYP2C8 in the metabolism of montelukast, which has not been fully characterised in humans.

As montelukast is excreted mainly in the bile, renal insufficiency is not expected to affect its pharmacokinetics. Patients with mild to moderate hepatic insufficiency had 41% higher mean montelukast area under the plasma drug concentration-time curve (AUC) following a single dose, and slightly prolonged elimination half-life ($t_{1/2}$). No dose adjustment are recommended based on this (Singulair Label 2012).

3.2 Drug interactions of montelukast

In vitro montelukast is a selective and potent CYP2C8 inhibitor: among 209 drugs screened for their ability to inhibit CYP2C8, it was the most potent inhibitor, with IC_{50} of 19.6 nM in HLMs and 9.2 nM in rhCYP2C8 (Walsky *et al.* 2005a). The inhibition mechanism was observed to be competitive with K_i values from 0.0092 to 0.15 μ M, and selective for CYP2C8 with 50-fold higher IC_{50} for the next potent interaction with CYP2C9 (Walsky *et al.* 2005b). In humans, however, montelukast does not inhibit CYP2C8 *in vivo*, which has been demonstrated in clinical studies with CYP2C8 substrates pioglitazone, rosiglitazone and repaglinide (Jaakkola *et al.* 2006a; Kajosaari *et al.* 2006; Kim *et al.* 2007).

Montelukast does not affect the pharmacokinetics of CYP3A4 substrate terfenadine or its metabolite fexofenadine or CYP2C9 and CYP3A4 substrate warfarin (Van Hecken *et al.* 1999; Singulair Label 2012). With regard to other drugs used in the treatment of asthma, montelukast does not significantly affect the pharmacokinetics of e.g. theophylline, prednisone, prednisolone or roflumilast (Böhmer *et al.* 2009; Singulair Label 2012). It has been administered with other drugs routinely used in the treatment of asthma without increase in adverse drug reactions (ADRs) (Singulair Label 2011).

With regard to the effect of other drugs on the pharmacokinetics of montelukast, the CYP3A4 inducer phenobarbital has decreased the AUC of a 10 mg single dose of montelukast by approximately 40%. No dose adjustment was recommended based on this finding (Holland *et al.* 1998; Singulair Label 2012).

In a recent study in healthy volunteers, administration of fluconazole to steady state (150 or 50 mg daily) decreased the AUC of montelukast by about 31% and 39%, respectively. Clarithromycin (1000 mg daily for two days), in turn, increased the montelukast AUC by 144%. The authors discussed that clarithromycin is a clinically important mechanism-based inhibitor of CYP3A4, and that the effects might also be due to alteration of transporter-mediated uptake of montelukast into hepatocytes. The effects of fluconazole were attributed to significant reduction of the bioavailability of montelukast due to competitive binding of fluconazole with organic anion-transporting polypeptide (OATP) or other transporters in the intestinal wall, or an increase of its clearance. (Hegazy *et al.* 2012)

Regarding potential for transporter-mediated drug interactions, in one study montelukast is suggested to be a substrate for OATP2B1 expressed on Madin-Darby canine kidney II (MDCKII) –cell monolayers (Mougey *et al.* 2009). It was suggested that OATP2B1 could contribute to the absorption of montelukast from the intestine, and when comparing the effect of a polymorphism of *SLCO2B1* (c.935G>A), it was associated with about 30% lower plasma concentration of montelukast in patients with the c.935GA genotype compared to those with the c.935GG genotype (Mougey *et al.* 2009). In a further study evaluating the interaction of montelukast with citrus juice, the c.935GA genotype was associated with reduced AUC and C_{max} of montelukast independent of the coingested juice (Mougey *et al.* 2011). In the combined data of both genotypes (c.935GG and c.935GA) the citrus juices had no significant effect on the pharmacokinetics of montelukast, compared to control. When the data was stratified by the c.935G>A genotype, orange juice resulted in a significant reduction in the AUC of montelukast in the c.935GG but not in the c.935GA genotypes (Mougey *et al.* 2011). The *in vitro* assays used in the study by Mougey *et al.* in 2009 to determine if montelukast is a substrate for OAT2B1 have been challenged in a further publication. They resulted in no direct evidence that montelukast is transported by OATP2B1 *in vitro*, or that the c.935G>A variant would alter that transport (Chu *et al.* 2012). In a study with healthy volunteers the *SLCO2B1* c.935GG, c.935GA and c.935AA genotypes had no effect on the pharmacokinetics of montelukast (Tapaninen *et al.* 2012).

Montelukast has also been suggested to inhibit multidrug resistance-associated protein 2 (MRP2), an efflux pump involved e.g. in the pharmacokinetics of taxol and saquinavir. In an *in vitro* study montelukast was a potent inhibitor of MRP2-mediated efflux of these substrates, suggesting adjunct therapy potential (Roy *et al.* 2009b).

3.3 Efficacy of montelukast

Montelukast reduces several markers of inflammation in patients with asthma: nitric oxide (NO) levels in exhaled air, sputum eosinophil levels, bronchial hyper-responsiveness and peak flow variability, as well as exercise-induced bronchoconstriction (Jatakanon *et al.* 1998; Leff *et al.* 1998; Reiss *et al.* 1998). When compared to placebo, it significantly increases the forced expiratory flow in one second (FEV₁) and reduces the daily β -agonist use in asthmatic patients (Reiss *et al.* 1996; Reiss *et al.* 1997). Intravenously administered montelukast provides rapid (15 minutes) improvement in FEV₁ that persists for 24 h (Dockhorn *et al.* 2000; Jarvis *et al.* 2000).

However, the efficacy of LTRAs, including montelukast, is modest when compared to inhaled corticosteroids. In a randomised, controlled trial of almost 900 patients with asthma, the average percentage change from baseline in FEV₁ was 13% with beclomethasone, 7% with montelukast and <1% with placebo. Beclomethasone had a greater clinical benefit than montelukast, but the asset of montelukast was faster onset of action and greater initial effect (Malmstrom *et al.* 1999). In a meta-analysis patients with mild to moderate asthma treated with LTRAs had a 65% increased risk of asthma exacerbation compared to those treated with inhaled corticosteroids. LTRA treatment was not associated with increased risk of withdrawals due to adverse effects. (Ducharme *et al.* 2004)

In symptomatic patients, addition of an LTRA is similar in effect as increasing the dose of inhaled corticosteroid with regard to e.g. improvement of the FEV₁ or risk of exacerbation.

However, addition of LABAs to inhaled corticosteroids results in statistically significantly better lung function improvement, reduction of the use of rescue β_2 -agonist and the number of symptom free days. (Ducharme *et al.* 2004; Ducharme *et al.* 2011)

LTRAs have a special niche in the treatment of aspirin sensitive asthma. About 10% of asthma patients suffer from 'aspirin insensitivity', more accurately a class-effect, non-steroidal anti-inflammatory drug (NSAID) hypersensitivity. These patients have increased production of cysteinyl leukotrienes, possibly due to e.g. upregulation of the 5-LO pathway or genetic disposition. When NSAIDs, by inhibiting the COX-enzymes, direct the AA metabolism away from the COX-pathway into the direction of the 5-LO pathway resulting in increased leukotriene synthesis, it further cascades into bronchoconstriction and mucus secretion in these patients (Dahlén *et al.* 2002; Knowles *et al.* 2007). In such patients the addition of montelukast has improved the pulmonary function and control of asthma markedly, over and above what was achieved by conventional controller treatment like corticosteroids and theophylline (Dahlén *et al.* 2002).

The numerous studies on the efficacy of LTRAs, mostly montelukast, have also been reflected in the treatment guidelines of asthma. Currently montelukast is positioned in the treatment of asthma in both adult and pediatric patients, as an option for inhaled corticosteroids in mild asthma, and in addition to inhaled corticosteroids (after addition of LABA) in moderate to severe asthma (British Guideline on the Management of Asthma 2008).

3.4 Safety of montelukast

Montelukast has been well tolerated in clinical studies. It has been administered in a very broad dose range, up to 90-fold the current recommended dose, without unexpected adverse effects (Reiss *et al.* 1996; Altman *et al.* 1998; Singulair Label 2012).

During clinical studies, the frequency of adverse events in patients receiving montelukast has been similar to that of patients on placebo. During the post-marketing phase several adverse effects have been reported in association to the use of montelukast, including allergic reactions, tremor, chest pain etc. (Jarvis *et al.* 2000). Also the possible psychiatric and behaviour-related ADRs of montelukast in pediatric patients have been under surveillance for some years. The typical adverse effects of inhaled corticosteroids, such as growth suppression, adrenal suppression and osteopenia have not been assessed in the clinical studies of montelukast, so that a clear comparison of the long-term safety between montelukast and corticosteroids is not possible (Ducharme *et al.* 2004).

4 Zafirlukast

Zafirlukast was the first LTRA introduced to the markets in the EU and the US in 1996. It is indicated for the prophylaxis and chronic treatment of asthma in adults and in children five years old or older. Like montelukast, zafirlukast is a selective, potent and competitive antagonist of the CysLT₁. It antagonised the binding of LTD₄ to CysLT₁ with an IC₅₀ ranging from 1.9 to 4.4 nM (Lynch *et al.* 1999; Sarau *et al.* 1999). In an *in vitro* comparison zafirlukast was 90-fold more potent than its metabolites in receptor antagonist activity (Accolate Label 2011). The characteristics and pharmacokinetic properties of zafirlukast are summarised in Table 4.1.

Table 4.1 Characteristics of zafirlukast

| Zafirlukast | |
|--|--|
| Chemical formula and name | C ₃₁ H ₃₃ N ₃ O ₆ S 4(5-cyclopentyloxy-carbonylamino-1-methyl-indol-3ylmethyl)-3-methoxy-N-o-tolylsulfonylbenzamide |
| Molecular weight | 575.7 |
| <i>In vitro</i> metabolism | CYP2C9: major pathway CYP3A4: reactive metabolite Minor metabolites: several CYP enzymes |
| <i>In vitro</i> inhibitory effect (HLMs) | CYP2C8: IC ₅₀ 0.38 µM CYP2C9: IC ₅₀ 7.0 µM CYP3A4: IC ₅₀ 20.9 µM CYP2C19, CYP1A2, CYP2D6 > 30 µM |
| Antagonism of LTD ₄ binding to CysLT ₁ | IC ₅₀ : 1.9–4.4 nM |
| Daily dose | 20 mg twice daily (≥ 12 y), 10 mg twice daily (5–11 y) |
| Oral bioavailability | Unknown, food reduces by 30–60% |
| Plasma protein binding | >99% (primarily albumin) |
| Volume of distribution | 70 l |
| t _{max} | 3 h |
| t _{1/2} | 10 h |
| Elimination | Metabolised 33% Metabolism and excretion in faeces (89%): CYP2C9 and CYP3A4 (based on <i>in vitro</i> studies) 10% in urine (metabolites) |
| Transporters | — |

(Accolate Pharmacology Review(s) 1998; Accolate Clinical Pharmacology and Biopharmaceutics Review(s) 1999; Lynch *et al.* 1999; Sarau *et al.* 1999; Shader *et al.* 1999; Dekhuijzen *et al.* 2002; Kassahun *et al.* 2005; Walsky *et al.* 2005a; Accolate Label 2011)

HLMs human liver microsomes
IC₅₀ inhibitor concentration producing 50% inhibition
LT leukotriene
CysLT₁ cysteinyl leukotriene receptor 1
t_{max} time to reach peak plasma concentration
t_{1/2} elimination half-life

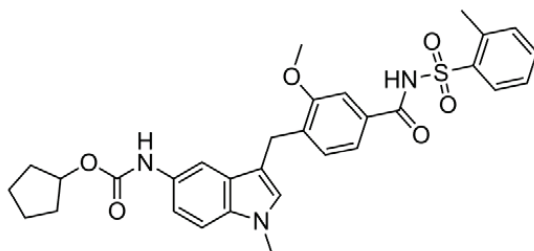
4.1 Metabolism of zafirlukast

According to its prescribing information, zafirlukast is extensively metabolised in humans, and the hydroxylated metabolites of zafirlukast excreted in the faeces are formed through CYP2C9 (based on *in vitro* studies), and 10% of orally administered radiolabelled zafirlukast is excreted in the urine and the remainder in faeces (Accolate Label 2011). According to further references 33% of zafirlukast is metabolised in humans and the main route of elimination is by metabolism and excretion in the faeces (89%), and 10% is excreted in the urine as metabolites (Accolate Pharmacology Review(s) 1998; Accolate Clinical Pharmacology and Biopharmaceutics Review(s) 1999). *In vitro*, CYP3A4 also participates in the biotransformation of zafirlukast, and in addition several CYP enzymes, e.g. CYP2C19, can catalyse the *in vitro* formation of some minor metabolites (Kassahun *et al.* 2005). The role of these CYP isoforms in the *in vivo* metabolism of zafirlukast in humans has, however, not been fully elucidated, as there are no published studies describing the human metabolism of zafirlukast.

According to some literature, the major biotransformation pathway of zafirlukast is hydroxylation by CYP2C9 (e.g. (Adkins *et al.* 1998; Dekhuijzen *et al.* 2002). However, the cited article describes the absorption, distribution, metabolism and excretion of radiolabelled zafirlukast in mice, rats and dogs (Savidge *et al.* 1998). The main component in dog bile was zafirlukast itself, accounting for 33% twelve hours after administration, and the metabolites were produced by five major reactions: cleavage of carbamate linkage, hydroxylations at various sites, *N*-demethylation at the indole nitrogen, *N*-acetylation at the 5-aminoindole position, and cleavage of the sulphonamide linkage (Savidge *et al.* 1998).

No differences in the pharmacokinetics of zafirlukast were observed between patients with impaired and normal renal function. In patients with hepatic impairment and cirrhosis the clearance of zafirlukast was reduced, resulting in a 50–60% increased AUC and peak plasma concentration (C_{max}). Zafirlukast is contraindicated in patients with hepatic impairment, including cirrhosis. (Accolate Label 2011)

Figure 4.1.1 Structure of zafirlukast



4.2 Drug interactions of zafirlukast

In vitro, zafirlukast inhibits several CYP enzymes, including CYP2C8, CYP2C9, CYP3A4, and CYP2C19, but only with much higher IC₅₀ than its clinically relevant free concentrations (Shader *et al.* 1999; Walsky *et al.* 2005a; Jaakkola *et al.* 2006b). Like montelukast it was among the most potent inhibitors of CYP2C8, with an IC₅₀ of 0.388 µM in HLMs and 0.644 µM in rhCYP2C8 (Walsky *et al.* 2005a). The IC₅₀ of zafirlukast for CYP2C9 inhibition was an order of magnitude higher, 7.0 µM (Shader *et al.* 1999). In humans, the pharmacokinetics of CYP2C8 substrate pioglitazone or CYP3A4 substrates clarithromycin and terfenadine were not significantly changed by zafirlukast in healthy volunteers (Garey *et al.* 1999; Dekhuijzen *et al.* 2002; Jaakkola *et al.* 2006a). Neither did zafirlukast have significant effect on the pharmacokinetics of oral contraceptives or theophylline (Accolate Label 2011), although isolated cases of increased theophylline levels after zafirlukast administration have been reported (Katial *et al.* 1998).

In healthy volunteers, the administration of zafirlukast (160 mg/day, four times the adult daily dose) to steady state resulted in over 60% increase in the S-warfarin AUC (single dose of 25 mg). Inhibition of CYP2C9 was the proposed mechanism of this interaction (Accolate Label 2011). The pharmacokinetics of zafirlukast did not change during the coadministration of warfarin. This interaction has warranted clinical caution, i.e. monitoring of the international normalised ratio (INR) due to the narrow therapeutic range of warfarin.

Coadministration of aspirin (2600 mg daily) with zafirlukast has resulted in a mean increase of 45% in zafirlukast plasma concentrations. When in turn single dose of zafirlukast was administered with erythromycin (500 mg three times daily to steady state), the plasma concentrations of zafirlukast decreased by about 40%, presumably due to decreased bioavailability of zafirlukast. Theophylline decreased the plasma concentrations of zafirlukast by 30–40% (Adkins *et al.* 1998; Dekhuijzen *et al.* 2002; Accolate Label 2011).

4.3 Efficacy of zafirlukast

Zafirlukast has been shown to reduce bronchoconstriction induced by several stimuli, including LTD₄, allergens, exercise, and cold air, sulphur oxide or platelet activating factor (Adkins *et al.* 1998; Dunn *et al.* 2001). In a study of over 700 patients treated for 13 weeks with zafirlukast or placebo, zafirlukast significantly increased the morning PEF and FEV₁, and decreased daytime asthma symptoms, night-time awakenings, mornings with asthma and β₂-agonist use, compared to placebo. The effects of zafirlukast appeared within two days (Fish *et al.* 1997). In a meta-analysis of five randomised and double-blind studies of zafirlukast, the risk of asthma exacerbation requiring withdrawal from zafirlukast was half of that of placebo, and similar for exacerbations requiring additional control medication and oral corticosteroids rescue (Barnes *et al.* 2000).

Compared to inhaled corticosteroids, zafirlukast is, like montelukast, less effective. When compared to fluticasone, fluticasone was significantly superior to zafirlukast, with regard to improvement in PEF and FEV₁, symptomatic improvement and less use of β₂-agonist use (Bleecker *et al.* 2000; Dunn *et al.* 2001). When compared to inhaled LABA salmeterol, salmeterol was also significantly more effective than zafirlukast in increasing the PEF values

and percentage symptom-free days in patients receiving concurrent inhaled corticosteroid (Busse *et al.* 1999).

However, the addition of zafirlukast to inhaled corticosteroid therapy has been at least as effective as doubling of the corticosteroid doses (Dunn *et al.* 2001). In line with this, zafirlukast is currently placed along with montelukast as a treatment alternative for both adult and pediatric asthma patients, as an option for inhaled corticosteroids in mild asthma, and in addition to inhaled corticosteroids (after addition of LABA), in moderate to severe asthma (British Guideline on the Management of Asthma 2008).

4.4 Safety of zafirlukast

In clinical studies the incidence and profile of adverse events have been similar between zafirlukast and placebo. The most frequent adverse event was headache in both treatment groups, followed by infection, nausea and diarrhoea (Adkins *et al.* 1998; Accolate Label 2011).

Severe hepatitis has been associated to zafirlukast treatment. The first cases reported were three female patients, one of whom eventually underwent liver transplantation (Reinus *et al.* 2000). Thereafter rare cases of fulminant hepatitis progressing to hepatic failure or death have been reported, and liver function testing is advised whenever signs or symptoms of liver dysfunction are present (Accolate Label 2011).

Zafirlukast and also montelukast, have been associated with Churg-Strauss syndrome, which is a rare form of vasculitis characterised by the presence of at least four of the following: asthma, eosinophilia, pulmonary infiltration, polyneuropathy, sinusitis and extravascular eosinophils. Within the first six months of zafirlukast being on the market, eight patients were reported to develop this condition while treated with zafirlukast, and several cases were reported in connection to montelukast as well (Wechsler *et al.* 1998; Wechsler *et al.* 1999). The most supported suggestion of the mechanism for this ADR is that the patients have an underlying eosinophilic disorder, presenting as asthma, and being masked by the corticosteroid therapy. Then the addition of a LTRA that allows the reduction of corticosteroids doses also allows the condition to become clinically apparent (Wechsler *et al.* 1998; Wechsler *et al.* 1999; Dunn *et al.* 2001).

5 Pharmacokinetics and drug metabolism

Pharmacokinetics is the science that describes what the body does to the drug, i.e. describes the relationship between the drug input and the concentration achieved with time. Pharmacodynamics in turn describes what the drug does to the body, i.e. the relationship between the drug concentration and the effects it produces, both desired and adverse. The desired and adverse effects of a drug are usually related to the concentration of the drug at the site(s) of action (Tozer and Rowland 2006).

Drugs can be administered extravascularly and intravascularly. When administered extravascularly, an extra step, absorption, is needed in order to enter the drug into the systemic circulation. Once the drug has reached the systemic circulation, it is delivered to all sites of the body, including the site where it exerts its effects and the organs that participate in its elimination, by metabolism and excretion. These phases of the drug in the body are defined as absorption and disposition. Disposition includes distribution and elimination, and elimination in turn includes metabolism and/or excretion. All these processes of absorption, distribution, metabolism and excretion (ADME) are influenced by several intrinsic and extrinsic factors such as age, race, gender, genetic polymorphisms, disease states as well as concomitant treatments and diet (Wilkinson 2001; Tozer and Rowland 2006; Holford 2009).

The absorption of an orally administered drug is affected by several factors, dosage form being the first. An orally administered solid dosage form drug needs to undergo disintegration or deaggregation and dissolution processes in order to reach solution form. Thereafter also physicochemical properties of the drug (e.g. ionisation), the physiological factors of the gastrointestinal tract (e.g. gastric emptying and intestinal motility), as well as gastrointestinal abnormalities and diseases (e.g. diarrhoea) all determine the absorption kinetics of the drug. Gastric emptying, membrane permeability and intestinal blood flow are among the most important determinants of the systemic absorption. (Tozer and Rowland 2006)

The term bioavailability describes the rate and the extent of the drug input to the systemic circulation. During the first-pass metabolism, an orally administered drug undergoes metabolism in the intestine wall and the liver before entering the systemic circulation. The portion that reaches the systemic circulation, e.g. bioavailability, is reduced. Enterohepatic cycle, in turn, is a cycle in which the drug may be excreted in the bile, stored in the gallbladder and released to the small intestine and from there reabsorbed again to the circulation. (Lin *et al.* 2001; Wilkinson 2005; Tozer and Rowland 2006)

The kidney and the liver are the two main organs of drug elimination: chemically unchanged drug or its metabolite(s) may be excreted in the urine or in the bile. Only a few drugs are eliminated almost entirely by excretion in the urine, whereas most drugs undergo biotransformation before they are excreted in the urine, bile or via other routes. Biotransformation by metabolism in the liver is the major mechanism for drug elimination from the body. (Krishna *et al.* 1994; Tozer and Rowland 2006)

5.1 Drug-metabolising enzymes

The biotransformation of drugs to metabolites aims to transform the drug from a lipid-soluble form to more water-soluble forms that are more easily extracted to urine and/or bile. The reactions of biotransformation are classified as phase I and phase II reactions (Figure 5.1.2.1). Phase I reactions, often occurring first, are also called functionalisation reactions and include oxidation, reduction, and hydrolysis. Phase II reactions, in turn, are conjugating reactions. In addition to this detoxifying effect, biotransformation may also be the mechanism for formation of active metabolites (Krishna *et al.* 1994; Meyer 1996; Pelkonen *et al.* 2005; Correia 2009; Huttunen *et al.* 2011).

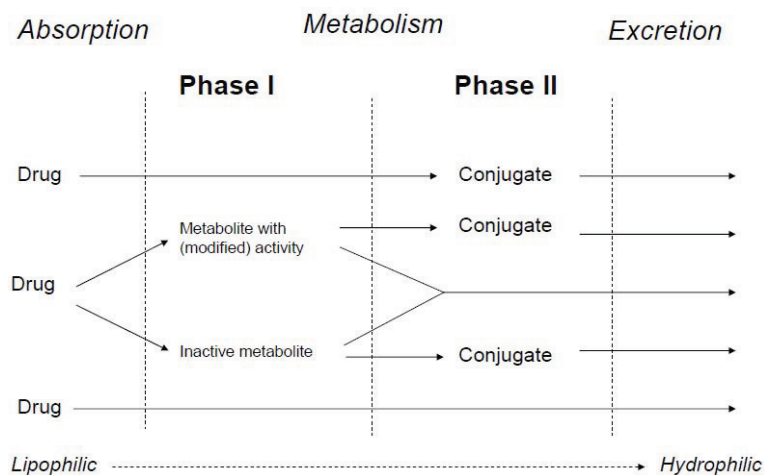
5.1.1 Phase I enzymes

The CYP enzymes are the most important enzymes of the phase I reactions in biotransformation of drugs (Figure 5.1.2.1), and also have a role in physiological homeostasis, e.g. bile acid synthesis, cholesterol metabolism and steroid and vitamin D synthesis and metabolism (Nebert *et al.* 2002; Estabrook 2003). They are a gene superfamily of heme-containing enzymes and present in many tissues, most abundantly in the liver and the gastrointestinal tract. It has been estimated that about ¾ of all drugs undergo oxidative metabolism catalysed by CYP enzymes, and about half of these are catalysed by CYP3A4 alone or partially (Wilkinson 2001; Bachmann *et al.* 2005; Johansson *et al.* 2011). The CYP enzymes are covered in more detail in section 7.

5.1.2 Phase II enzymes

Phase II reactions, i.e. conjugation, occur when either the parent drug, or its phase I metabolite, containing a suitable chemical group (resulting from the phase I reaction) and the appropriate transferase enzyme are present to yield a highly polar conjugate that is rapidly excreted (Figure 5.1.2.1). The transferase enzymes are located in the cytosol or microsomes. Most important of these are the products of the uridine 5'-diphosphate (UDP) glucuronosyltransferase (UGTs) gene family, responsible for about 35% of all phase II reactions (Evans *et al.* 1999; Guillemette 2003; Miners *et al.* 2010). Other enzymes catalysing phase II reactions include e.g. sulfotransferases (STs), glutathione S-transferases (GSTs), involved e.g. in the metabolism of leukotrienes, as well as *N*-acetyltransferases (NATs), methyltransferases (MTs) and epoxide hydrolases (EHs) (Wilkinson 2001; Evans *et al.* 2003; Correia 2009).

Figure 5.1.2.1 Elimination of drugs by metabolism and/or direct excretion. Phase II reactions may also occur before phase I reactions (adapted from Correia 2009)



5.2 Transporters

In addition to passive diffusion processes, the ADME processes of an orally administered drug may also include active membrane transporters. Membrane transporters are proteins expressed on the apical or basolateral side of epithelial cells in various tissues, mediating the passage of chemicals, including drug molecules, into and out of cells. For example, in the hepatocytes transporters participate in the uptake, sinusoidal efflux and biliary excretion of drugs. Thus a transporter can affect the pharmacokinetics of a drug, either alone or complementing pharmacokinetic processes by e.g. extracting drugs from the portal blood into hepatocytes, to the site of biotransformation in phase I and II reactions. Similarly, in renal epithelial cells, transporters are involved in tubular secretion and reabsorption. (Shitara *et al.* 2006; Noe *et al.* 2007; Zhang *et al.* 2008; Kalliokoski *et al.* 2009; Niemi *et al.* 2011)

The understanding of the role and importance of transporters in pharmacokinetics, drug response, and drug interactions have vastly increased during the past decades. According to current knowledge over 400 membrane transporters exist, and are divided into two major superfamilies, adenosine triphosphate (ATP) binding cassette and solute carrier. Both these superfamilies include marked generic variability in the coding region. (Huang *et al.* 2008; Zhang *et al.* 2008; Yee *et al.* 2010; Niemi *et al.* 2011)

Examples of hepatic transporters and their substrate drugs include P-glycoprotein (P-gp) and digoxin, organic anion-transporting polypeptides (OATP) 1B1, 1B3 and 2B1 and rosuvastatin, as well as breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 2 (MRP2) and pravastatin, pitavastatin and rosuvastatin. (Shitara *et al.* 2006; FDA Draft Guidance for Industry 2012)

5.2.1 P-gp/MDR1

P-gp or multidrug resistance protein 1 (MDR1) is the most studied ATP-binding cassette transporter. In humans there are two MDRs encoded by *MDR1* and *MDR3* genes, of which only the *MDR1* gene product participates in drug disposition. P-gp is localised in canalicular surface of hepatocytes, apical surface of renal tubular cells, as well as in the intestine, placenta and capillary endothelial in the brain. It is able to transport many structurally divergent compounds, and it functions as an efflux transporter, translocating drugs out of cells to the extracellular compartment. The substrate and inhibitor specificity of P-gp overlaps significantly, but not absolutely with CYP3A4/5, and the complementary functions of these proteins may reduce the systemic exposure of their substrate drugs. Drug interactions resulting from inhibition of P-gp in the liver, intestine and kidney have been recognised, e.g. interaction of digoxin with known P-gp inhibitors, e.g. quinidine, itraconazole and cyclosporine. (Kim 2002; Lin 2003)

5.2.2 OATPs

OATPs are membrane influx transporters encoded by the solute carrier organic anion transporter (*SLCO*) gene family. Three members of this family, OATP1B1, OATP1B3 and OATP2B1, are expressed in sinusoidal membrane of hepatocytes, and facilitate the liver uptake of their substrates. Both drug interactions inhibiting these transporters and genetic variability in the *SLCO* genes encoding them result in significant interindividual differences in the pharmacokinetics of their substrates and even susceptibility to ADRs. For example, in a

genome-wide association study the *SLCO1B1* c.521T>C single nucleotide polymorphism (SNP) was the only strong marker associated with simvastatin-induced myopathy. (Noe *et al.* 2007; Link *et al.* 2008; Kalliokoski *et al.* 2009; Niemi *et al.* 2011)

6 Drug interactions

Drug interactions are a considerable cause for morbidity. They may lead to ADRs, which in turn may lead to both hospital admissions and prolongation of the stay in the hospital (Pirmohamed *et al.* 2004; Huang *et al.* 2008). ADRs have been estimated to lead to hospital admission in about 7% of all admissions (Pirmohamed *et al.* 2004; Hofer-Dueckelmann *et al.* 2011), and 7–15% of in-hospital patients have been estimated to experience ADRs during their hospital stay (Lazarou *et al.* 1998; Pirmohamed *et al.* 2004; Davies *et al.* 2009). In studies conducted in Finland 2.3% of visits to a district hospital emergency room were drug-related, including intentional overdoses (Juntti-Patinen *et al.* 2006).

Drug interactions and ADRs may also result in fatal outcomes, and in a Finnish study 5% of deaths in a university hospital were assessed as drug-related (Juntti-Patinen *et al.* 2002). In a multivariate analysis the only significant predictor for ADRs was the number of drugs the patient was taking (Davies *et al.* 2009), and ADRs most frequently occurred with diuretics, opioid analgesics, anticoagulants and NSAIDs (Pirmohamed *et al.* 2004; Davies *et al.* 2009; Hofer-Dueckelmann *et al.* 2011). Between 1999 and 2003 half of the drug withdrawals from the market in the US due to safety reasons were associated with important drug interactions (Huang *et al.* 2008).

6.1 Mechanisms of drug interactions

Drug interactions arise in numerous ways, including both pharmacokinetic and pharmacodynamic mechanisms. Pharmacodynamic interactions may result when drugs e.g. compete with each other at the level of the receptor, cell membrane or ion channel, or exert similar or opposing pharmacodynamic (therapeutic or adverse) effects (EMA Guideline on the investigation of Drug Interactions (final) 2012). Examples of these include e.g. increasing risk of Torsades de Pointes (TdP) -type of cardiac arrhythmia with drugs that prolong the QT-time, increased risk of gastro-intestinal bleeding with concomitant NSAIDs and selective serotonin reuptake inhibitors (SSRIs), or decreased or absent effect of β_2 -agonist if administered concomitantly with an unselective β -blocker.

Pharmacokinetic interactions may affect the absorption, distribution, metabolism or elimination of the drug (Table 6.1.1). Especially when metabolic routes of elimination, in particular those catalysed by the CYP enzyme family, are inhibited or induced by another drug, even hundred fold differences in the exposure to the drug may occur (Backman *et al.* 1998; Huang *et al.* 2007). Also interindividual differences and genetic polymorphism of e.g. drug metabolising enzymes or transporters contribute to the variability seen in drug concentrations, therapeutic doses and clinical responses between patients.

Table 6.1.1 Examples of pharmacokinetic interaction mechanisms

| Phase | Mechanism (change in) | Example | Reference |
|---------------------|--|---|--------------------------------|
| Absorption | Gastric pH | Tolfenamic acid-MgOH (tolfenamic acid solubility↑, AUC ↑) | (Neuvonen <i>et al.</i> 1988) |
| | Gastric/intestinal motility | Metoclopramide-digoxin (motility ↑, digoxin [C] ↓) | (Manninen <i>et al.</i> 1973) |
| | Physicochemical (formation of chelates, adsorption by other drug) | Iron-tetracycline (tetracycline bioavailability ↓) | (Neuvonen <i>et al.</i> 1970) |
| | First-pass metabolism (induction or inhibition of metabolising enzyme) | Grapefruit juice-felodipine (CYP3A4 inhibition, felodipine AUC ↑) | (Bailey <i>et al.</i> 1991) |
| | Transport mechanisms in the small intestine | Itraconazole-aliskiren (inhibition of P-gp, aliskiren AUC, C_{max} ↑) | (Tapaninen <i>et al.</i> 2011) |
| Distribution | Binding to plasma proteins | ASA-phenytoin (protein binding displacement of phenytoin)* | (Leonard <i>et al.</i> 1981) |
| | Distribution to tissues (transport mechanisms) | Ketoconazole-ritonavir (inhibition of P-gp at BBB, ritonavir [C] in CSF ↑) | (Khaliq <i>et al.</i> 2000) |
| | Hepatic uptake (transport mechanisms) | Cyclosporine-pravastatin (inhibition of OATP1B1, pravastatin AUC, C_{max} ↑) | (Hedman <i>et al.</i> 2004) |
| Metabolism | Activity of metabolising enzyme | Gemfibrozil-repaglinide (CYP2C8 inhibition, repaglinide AUC, C_{max} ↑) | (Niemi <i>et al.</i> 2003b) |
| | | Rifampicin-midazolam (CYP3A4 induction, midazolam AUC, C_{max} ↓) | (Backman <i>et al.</i> 1996a) |
| Excretion | Biliary excretion (transport mechanisms) | Erythromycin-ximelagatran (inhibition of P-gp, melagatran AUC, C_{max} ↑) | (Eriksson <i>et al.</i> 2006) |
| | Intestinal excretion (transport mechanisms) | Verapamil-talinolol (i.v.) (inhibition of intestinal P-gp, decreased active secretion of talinolol) | (Gramatte <i>et al.</i> 1999) |
| | Urine pH | Amphetamine-NaHCO ₃ (alkalisation of urine, excretion of amphetamine ↓) | (Beckett <i>et al.</i> 1965) |
| | Renal blood flow | Indomethacin-lithium (renal blood flow ↓, lithium CL_R ↓, [C] ↑) | (Frölich <i>et al.</i> 1979) |
| | Renal excretion (transport mechanisms) | Itraconazole-digoxin (inhibition of P-gp, digoxin CL_R ↓, AUC↑) | (Jalava <i>et al.</i> 1997b) |
| | | Phenytoin-digoxin (induction of P-gp, digoxin CL_R ↑, AUC ↓) | (Rameis 1985) |

| | |
|--------------------|--|
| * | minor or no clinical relevance |
| MgOH | magnesiumhydroxide |
| AUC | area under the plasma drug concentration-time curve |
| [C] | plasma/serum concentration |
| P-pg | P-glycoprotein (=multiple drug resistance protein 1, MDR1) |
| C _{max} | peak plasma concentration |
| ASA | acetylsalicylic acid |
| BBB | blood-brain barrier |
| CSF | cerebrospinal fluid |
| OATP | organic anion-transporting polypeptide |
| i.v. | intravenous |
| CL _R | renal clearance |
| NaHCO ₃ | sodiumbicarbonate |

6.2 Inhibition of metabolising enzyme

Inhibition of a metabolising enzyme is the most common mechanism for inhibitory drug interactions, and inhibition of CYP enzymes accounts for approximately half of all pharmacokinetic interactions (Bachmann *et al.* 2005). Inhibition of the metabolising enzyme may lead to decreased clearance (CL) and increased exposure (reflected by AUC) of the parent drug and decreased concentrations of the metabolite(s). This further leads to prolonged and enhanced pharmacological effects, and to increased likelihood for concentration-dependent ADRs and toxicity, the clinical consequences being most critical for drugs with a narrow therapeutic range. On the other hand, therapeutic effect of a prodrug decreases. In general, a less than twofold increase in the AUC usually does not produce clinical consequence, whereas greater than twofold increase warrants concern for clinical consequences.

The inhibition of a metabolising enzyme occurs by two main mechanisms: reversible inhibition and mechanism-based inhibition. Reversible inhibition may be competitive, non-competitive, uncompetitive or mixed-type, whereas mechanism-based inhibition occurs as quasi-reversible or irreversible. (Segel IH 1975; Kalgutkar *et al.* 2007; Obach *et al.* 2007; Zhou 2008)

Most of the CYP-mediated reactions follow the simple Michaelis-Menten (M-M) kinetics, which represents a simplified model of a single binding site. For reactions following the M-M kinetics the substrate M-M kinetic constant (K_m), describing the substrate concentration at which the reaction rate is at half-maximal velocity, and the maximum velocity (V_{max}) can be delivered. The M-M model assumes that the active site of the enzyme contains one binding site, and the velocity of the reaction can be characterised as a hyperbolic saturating curve (velocity vs. substrate concentration). When the reaction is then inhibited by an inhibitor, a value describing the inhibitor concentration supporting half of the maximal inhibition (K_i) can be determined. (Segel IH 1975; Shou *et al.* 2000; Zhang *et al.* 2005)

In vitro, several CYP isoforms, including CYP3A4 and CYP2C9 do not always follow the above kinetics, but atypical, or non-Michaelis-Menten kinetics, representing a model of two or more binding regions in the active site(s), resulting in non-hyperbolic velocity curve. Atypical kinetics include a wide range of situations including substrate activation or inhibition, and partial activation or inhibition (Shou *et al.* 2000; Atkins 2005; Zhang *et al.* 2005). However, examples

of non-M-M kinetics *in vivo* are so far few. The flavonoid tangeretin, for example, activated regioselectively the CYP3A4-mediated metabolism of midazolam *in vitro*, whereas tangerine juice, which contains a high amount of tangeretin, did not affect the metabolism of midazolam in humans (Backman *et al.* 2000b). Recently it has been shown, however, that fluconazole affected the metabolism of midazolam both *in vivo* and *in vitro*, in a manner consistent with an allosteric interaction (Yang *et al.* 2012).

6.2.1 Reversible inhibition

Reversible inhibition may be competitive, non-competitive, uncompetitive, or mixed inhibition. In competitive inhibition the inhibitor binds only to an unbound enzyme. It occurs when two or more substrate drugs compete for the same active site of the enzyme, i.e. the enzyme active site is also the inhibitor-binding site, and the inhibitor is non-covalently bound to the enzyme. The extent of the competition depends on the relative concentrations of the substrate/inhibitor drugs and their affinity to the active site. Competitive inhibitor increases the substrate's K_m without affecting the V_{max} of the reaction, and the IC_{50} increases with the increase in the substrate concentration. Competitive inhibition starts rapidly and ends with the clearance of the inhibitor from the body, or can be overcome by increasing the concentration of the substrate. It is the prevalent form of reversible inhibition. (Segel IH 1975; Shou *et al.* 2000; Zhang *et al.* 2005; Blat 2010)

In non-competitive inhibition the inhibitor binds to both an unbound and a substrate bound enzyme with the same binding affinity, but the substrate and the inhibitor are binding to different sites of the enzyme. It may also occur when the inhibitor causes conformational changes to the active site that prevents substrate binding, i.e. the inhibitor and substrate bind to the same site but at different time points. The non-competitive inhibitor decreases the V_{max} without affecting the K_m and the substrate concentration does not affect the inhibitor IC_{50} . (Segel IH 1975; Shou *et al.* 2000; Zhang *et al.* 2005; Blat 2010)

In uncompetitive inhibition the inhibitor binds only to a substrate bound enzyme. It is the rarest form of reversible inhibition. Mixed inhibition involves a combination of two or more of the above mechanism of reversible inhibition, particularly the combination of competitive with non-competitive. (Zhang *et al.* 2005; Fowler *et al.* 2008)

6.2.2 Mechanism-based inhibition

Mechanism-based inhibitors are further sub-divided into irreversible or quasi-irreversible inhibitors. They both require at least one catalytic cycle of the CYP catalysed process, and are dependent on time, concentration and energy (NADPH), catalysing the formation of a reactive intermediate. (Silverman 1995; Kalgutkar *et al.* 2007)

In a quasi-irreversible inhibition the reactive intermediate interacts non-covalently with the heme iron of the prosthetic group of the CYP enzyme, leading to a formation of an inactive metabolite-inhibitor complex and an inactive state of the enzyme. In an irreversible inhibition the reactive intermediate covalently reacts with the active site amino acid residue of the apoprotein and/or directly destroys the heme prosthetic group, leading to permanent loss of the enzyme function. Thus, this irreversible form of mechanism-based inhibition is also called 'suicide-inhibition'. (Rando 1984; Silverman 1995; Kalgutkar *et al.* 2007)

The mechanism-based inhibition, compared to the reversible inhibition, more frequently results in unfavourable drug interactions. The onset of the inhibition may be rapid, and it persists longer, as the inactivated enzyme function is regained only by the newly synthesised protein. One well characterised example of a mechanism-based inhibitor is the glucuronidated metabolite of gemfibrozil, which irreversibly inactivates CYP2C8, resulting in a long-lasting inhibition of it (Ogilvie *et al.* 2006; Tornio *et al.* 2008a; Backman *et al.* 2009).

6.3 Induction of metabolising enzyme

An inducer is a drug that increases the metabolic activity of a CYP enzyme by increasing its synthesis (up-regulation of gene expression or stabilisation of mRNA) or by decreasing its catabolism (post-translational stabilisation). This induction of the metabolising enzyme results in a decrease of plasma concentration and the pharmacological effect of the substrate drug, or increased effect in case of a pro-drug. Due to the transcriptional process, enzyme induction takes longer to develop than inhibition, i.e. approximately 12 days, or 1–5 days in case of autoinduction. Enzyme induction reaches its peak in 1–3 weeks and vanishes within 1–4 weeks after discontinuation of the inducer drug, but demonstrates large interindividual variability in the changes of the enzyme level. (Lin *et al.* 2001; Wilkinson 2001; Zhou *et al.* 2008)

The underlying mechanisms of the induction of the drug metabolising CYP families 1–3 have been elucidated in more detail over the past decades along the increase in the database of known CYP genes. In the 1980s it was already known that the induction of CYPs involves activation of the genetic information, but only later the nuclear receptor mechanisms have been uncovered. Most, but not all genes of the CYP families 1–3 can be transcriptionally activated by foreign chemicals that induce gene expression through receptor dependent mechanisms. (Bresnick *et al.* 1984; Zhou 2008)

The inducer effect of polycyclic hydrocarbon in rats was among the first reports of induction of CYPs in the 1950s (Richardson *et al.* 1951; Bresnick *et al.* 1984). Transcription of CYP1 genes is induced by the aryl hydrocarbon (Ah) receptor, which is activated by an aromatic hydrocarbon ligand. Thus CYP1 genes are induced by large number of polycyclic aromatic hydrocarbons, including environmental carcinogens such as cigarette smoke (Zhu 2011).

CYP families 2–3 are induced by the same nuclear receptor gene family members: constitutive androstane receptor (CAR), pregnane X receptor (PXR) and peroxisome proliferator-activated receptor (PPAR). Simplified, the nuclear receptors are activated by the conformational changes caused by the binding of the ligand to the receptor, and then get the ability to bind directly to deoxyribonucleic acid (DNA) segments and regulate the expression of adjacent genes. A well-established inducer of CYP3A4 is rifampicin, acting through transcriptional up-regulation. (Waxman 1999; Zhou 2008)

6.4 Drug interactions and drug development

As a crude estimate one in 10000 new candidate molecules being screened for further development results in a drug that reaches a marketing authorisation. A failure in drug development becomes increasingly costly the later in development it occurs, most costly when

resulting in withdrawal from the market after marketing approval. Therefore it could even be said that the primary aim of drug development is to cease the development of a poor candidate molecule showing a poor risk-benefit profile in as early phase of the development as possible. If not killable, the vulnerability of the pharmacokinetics and the potential for drug interactions also become determinates that could affect the profitability of the candidate molecule for further development. Thus it is increasingly important to understand the drug candidate's potential for interactions as early in the development as possible.

Prediction of clinically significant drug interactions during drug development is a challenge for both the pharmaceutical industry and the regulatory authorities. Both the Food and Drug Administration (FDA) and European Medicines Agency (EMA) have guidelines on the investigation of drug interactions, from the early *in vitro* screening to the *in vivo* studies and the possible implications of an observed interaction to the product information. As mentioned, between 1999 and 2003 half of the drug withdrawals in the US that occurred after achieving the marketing authorisation were due to drug interactions resulting in serious ADRs (Table 6.4.1) (Huang *et al.* 2008).

Table 6.4.1 Examples of drug withdrawals due to drug interactions

| Withdrawn drug and year | Interaction mechanism | Clinical consequence | Reference |
|-------------------------|--|----------------------|--|
| Terfenadine 1998 | Inhibition of metabolism by CYP3A4 inhibitors | QT prolongation, TdP | (Monahan <i>et al.</i> 1990) |
| Mibefradil 1998 | Inhibits metabolism of astemizole, cisapride, and terfenadine via CYP3A4 | QT prolongation, TdP | (Ernst <i>et al.</i> 1998; Dresser <i>et al.</i> 2000) |
| Astemizole 1999 | Inhibits metabolism of simvastatin and lovastatin via CYP3A4 | Rhabdomyolysis | (Schmassmann-Suhjar <i>et al.</i> 1998) |
| | Inhibition of metabolism by CYP3A4 inhibitors | QT prolongation, TdP | (Dresser <i>et al.</i> 2000) |
| Cisapride 2000 | Inhibition of metabolism by CYP3A4 inhibitors | QT prolongation, TdP | (Dresser <i>et al.</i> 2000) |
| Cerivastatin 2001 | Inhibition of metabolism by gemfibrozil | Rhabdomyolysis | (Chang <i>et al.</i> 2004) |

TdP Torsades de Pointes

In the beginning of the 1990s an interaction between terfenadine and CYP3A4 inhibitors was reported, resulting in TdP, a life-threatening cardiac arrhythmia (Monahan *et al.* 1990; Bode 2010). This was one of the initiating factors for the development of guidelines for studying drug interactions. Both the EMA and the FDA published their first guidelines in 1997 and these guidelines have been updated since then to reflect the scientific advances (Huang *et al.* 2008). The need to include CYP2C8 in the evaluation of interactions was addressed by the FDA in 2003, following e.g. the interaction observed between gemfibrozil and cerivastatin, resulting in rhabdomyolysis (Backman *et al.* 2002; Chang *et al.* 2004; Huang *et al.* 2008). The EMA guideline on the investigation of drug interactions has been recently revised. The draft update has been available since 2010 and the final version will come into effect in January 2013 (EMA Guideline on the investigation of Drug Interactions (final) 2012).

Along the reaction phenotyping, i.e. characterisation of the specific enzymes responsible for the metabolism of the drug under investigation, the target of the drug interaction studies during drug development is to explore whether the candidate drug affects other drugs, or *vice versa*, and whether this leads to clinically relevant consequences warranting dose adjustment or therapeutic drug monitoring (TDM). Interaction potency should be considered at all phases of pharmacokinetics (ADME). Also it should be studied if the candidate drug affects the metabolism of drugs already on the market that are likely to be administered concomitantly, or *vice versa*. (EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012)

This translates to investigations using HLMs that aim to identify the enzyme(s) that metabolise the candidate drug. Especially if the drug is metabolised by one enzyme, a clinical study to explore the effect of inhibition of this enzyme *in vivo* is needed. In addition, the candidate drug's potential to induce or inhibit drug metabolising enzymes is studied *in vitro*. If the inhibition or induction potency is similar to the plasma concentrations achieved *in vivo*, or predictions produced by e.g. physiologically based pharmacokinetic (PBPK) modelling suggest interaction potential, a clinical study is needed, using a characterised probe substrate for that enzyme.

Table 6.4.2. Examples of *in vitro* marker reactions and *in vivo* probe drugs, inhibitors and inducers for specific CYP enzyme activities (adapted from EMA Guideline on the Investigation of Drug Interactions (final) 2012 and FDA Draft Guidance for Industry 2012)

| CYP | <i>In vitro</i> marker reaction | <i>In vivo</i> probe | <i>In vivo</i> inhibitor | <i>In vivo</i> inducer |
|---------|--|---|--|---|
| 1A2 | phenacetin-O-deethylation | caffeine, theophylline, tizanidine | ciprofloxacin, enoxacin, fluvoxamine | phenytoin ¹ , smoking ¹ |
| 2C8 | paclitaxel 6-hydroxylation amodiaquine N-deethylation | repaglinide ² | gemfibrozil ³ | rifampicin ¹ |
| 2C9 | S-warfarin 7-hydroxylation diclofenac 4'-hydroxylation | celecoxib, S-warfarin, tolbutamide | fluconazole ^{1, 3} | carbamazepine ¹ , rifampicin ¹ |
| 2C19 | S-mephenytoin 4'-hydroxylation | lansoprazole, omeprazole, | fluconazole ³ , fluvoxamine ³ , ticlodipine ³ | rifampicin ¹ |
| 2D6 | bufuralol 1'-hydroxylation | atomoxetine, desipramine, metoprolol, nevilolol, tolterodine, venlafaxine | bupropion, fluoxetine, paroxetine, quinidine | none identified |
| 3A4/3A5 | midazolam 1-hydroxylation testosterone 6β-hydroxylation | budesonide, buspirone, fluticasone, lovastatin, midazolam, simvastatin, triazolam | clarithromycin, itraconazole, ketoconazole, ritonavir | carbamazepine, phenytoin, rifampicin |

1 moderate inhibitor/inducer

2 also a substrate of OATP1B1

3 inhibits also other CYP enzymes / transporters

When focusing on interaction studies with CYP enzymes, the guidelines include examples of recommended or well validated marker reactions for specific CYP enzyme activities *in vitro*, as well as recommended *in vivo* probe drugs for specific CYP enzyme activities (Table 6.4.2). The guidelines also include some rules of thumb for defining the significance of the observed interaction. A twofold increase in the AUC is usually considered clinically significant, and for drugs with a narrow therapeutic range even a 50% increase in the AUC may be important and warrant dose adjustments. The potency of the inhibitor drug can also be graded: if the drug causes a fivefold or larger increase in the AUC of the probe substrate, or 80% decrease in the clearance, it is considered a strong inhibitor of that CYP enzyme. If it increases the AUC twofold to fivefold, or decreases the clearance 50–80%, it is a moderate inhibitor, and if the increase in the AUC is between 1.25 and twofold, or the decrease in the clearance is 20–50%, it is a weak inhibitor of that CYP enzyme. (Huang *et al.* 2007; EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012)

7 CYP enzymes

The CYP enzymes were first reported in 1958 as an unknown carbon monoxide-binding pigment in rat liver microsomes, which absorbed the wavelength 450 nm of light in reduced form combined with carbon monoxide (Klingenberg 1958). During the first half of the 1960s the hemoprotein nature of this pigment was characterised and the name P450 was proposed (Omura *et al.* 1962; Omura *et al.* 1964; Omura 1999). At that time the function and role of this pigment in the oxidation of drugs by liver microsomes was also discovered (Estabrook *et al.* 1963; Cooper *et al.* 1965; Estabrook 2003).

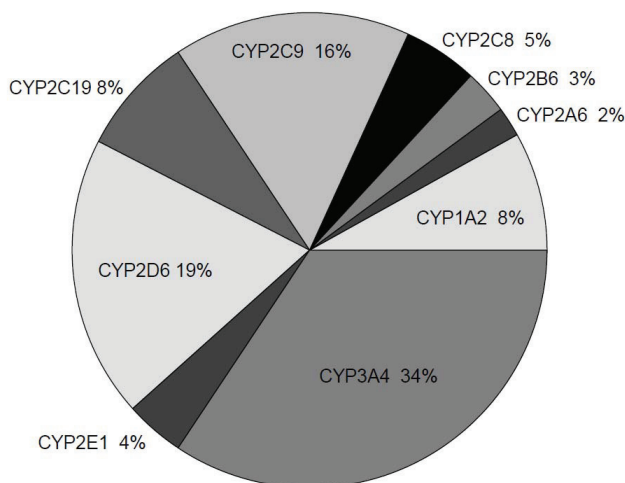
Since then a continually increasing amount of data on CYP enzymes has been published. It has been found in all eukaryotic organisms, animals, plants and fungi, and nowadays there are at least 270 different CYP gene families, 18 in mammals, possibly having a single ancestral gene before the time of prokaryote/eukaryote divergence. Along the expansion of the number of CYP enzymes recognised, a systematic classification system based on the similarity of the amino acid sequences was proposed in 1987, with a fifth update published in 1996 (Nelson *et al.* 1996; Omura 1999; Nebert *et al.* 2002).

The CYP enzyme gene superfamily and its products are arranged in families and subfamilies based on the amino acid sequence identity percentage: enzymes sharing $\geq 40\%$ identity form a family designated by an Arabic number (e.g. CYP3), enzymes sharing $\geq 55\%$ identity form a subfamily designated by a letter (CYP3A), and the individual gene or enzyme indicated by a number (CYP3A4). The same numbers and letters are recommended for the corresponding gene (*italics*) and gene product (non-*italics*) (Nelson *et al.* 1996; Nebert *et al.* 2002). The human genome contains 115 CYP genes, with 57 functional and the remaining 58 non-functional pseudogenes (Johansson *et al.* 2011).

The CYP enzymes are important in the oxidative, peroxidative and reductive metabolism of various endogenous compounds, including e.g. steroids, bile acids and fatty acids. In addition they participate in detoxification of foreign chemicals (xenobiotics), and the CYP families 1–3

account for approximately 75% of all phase I drug metabolism reactions. The contribution of different CYP families to drug metabolism is presented in Figure 7.1.

Figure 7.1. Involvement of different CYPs in drug oxidations (Evans *et al.* 1999; Daly 2004; Totah *et al.* 2005)



The reaction cycle of a CYP enzyme includes a flavin domain that uptakes and transfers reducing equivalents from NADPH or NADH, as well as a heme domain in which the substrate oxidation occurs. Eucaryotic CYP enzymes are integral membrane proteins of the endoplasmic reticulum or the inner mitochondrial membrane (Omura 1999; Vaz 2001).

The level of expression and catalytic activity of the CYP enzymes participating in drug metabolism varies considerably between individuals, resulting from both environmental and genetic factors. The genetic factors include genetic polymorphism of coding and regulatory sequences of *CYP* genes, as well as less well understood polymorphism of e.g. transcription factors. Genetic polymorphism refers to the occurrence of two or more genetically determined phenotypes in the same population in such proportions that the rarest of them cannot be maintained merely by recurrent mutation. Genetic polymorphism most commonly results from single nucleotide polymorphism (SNP), but also from deletion or insertion of base pairs, or copy number variations of the gene. (Daly 2004; Daily *et al.* 2009; Rodriguez-Antona *et al.* 2010; Johansson *et al.* 2011)

CYP polymorphism was first observed in the 1970s, when wide interindividual range in plasma concentrations of antihypertensive debrisoquine and antiarrhythmic sparteine were observed, leading to adverse effects in individuals with high concentrations. This observation was a consequence of the metabolism of these drugs by *CYP2D6*, with *CYP2D6* showing the highest degree of polymorphism of drug metabolising CYPs. *CYP2A6*, *2B6*, *2C8*, *2C9* and *2C19* are also polymorphic with various degree of functional significance, while *CYP1A2*, *2E1* and *3A4*

show no important functional polymorphism, except for the expression of *CYP3A5* (Daly 2004; Johansson *et al.* 2011). As a result of the variability in genes encoding CYP enzymes, populations can be classified into four major phenotypes, i.e. ultrarapid metabolisers (UM) with more than two active genes encoding a certain CYP, extensive metabolisers (EM) with two functional genes, poor metabolisers (PM) with lack of functional enzyme due to defective or deleted genes, as well as intermediate metabolisers (IM) with usually one functional and one defective allele, or two partially defective alleles (Ingelman-Sundberg *et al.* 2007).

7.1 CYP2C8

Although CYP2C8 was purified from the human liver in 1987 (Lasker *et al.* 1987), the knowledge of its quantum in drug metabolism was lagging the other CYP enzymes past the Millennium. The interaction of gemfibrozil and cerivastatin resulting in even fatal cases of rhabdomyolysis brought attention to the importance of CYP2C8 in drug metabolism (Chang *et al.* 2004; Neuvonen *et al.* 2006). This knowledge has since been increasing along the elucidation of the structure of CYP2C8, characterisation of clinically relevant substrates and polymorphism of the *CYP2C8* gene.

The human *CYP2C* gene cluster is located in the chromosome 10q24 with the *CYP2C* genes in the following order: *CYP2C8-CYP2C9-CYP2C19-CYP2C18*. The *CYP2C8* gene was cloned in 1999, and it spans 31 kilobases and consists of nine exons, being the smallest of human *CYP2C* genes (Gray *et al.* 1995; Klose *et al.* 1999).

CYP2C8 is mainly expressed in the liver, and was estimated in 2005 to contribute about 7% of the total microsomal CYP content, and to account for the oxidative drug metabolism by phase I enzymes of about 5% of drugs (Figure 7.1) (Totah *et al.* 2005). Its mRNA has also been detected in several extrahepatic tissues, including kidney, intestine, adrenal gland, brain, mammary gland, ovary, heart and breast cancer tumors (Klose *et al.* 1999). Extrahepatic CYP2C8 protein has been detected by immunohistochemistry in the kidney (proximal and distal tubulus and collecting duct), the small and large intestine, the adrenal cortical cells, the salivary ducts and the tonsils (Enayetallah *et al.* 2004). The CYP2C8 detected in the kidney and the expression of *CYP2C8* and *CYP2C9* in the endothelial cells of the heart, the aorta and the cardiac vessels has also been linked to metabolism of AA to EETs by CYP epoxigenases. The EETs are suggested to have several functions in cardiovascular system in addition to maintenance of vascular tone, such as inhibition of endothelial activation, inhibition of hemostasis, and protection against myocardial ischemia reperfusion injury (Zeldin *et al.* 1995; Totah *et al.* 2005; Chen *et al.* 2009; Deng *et al.* 2010).

7.1.1 Structure of CYP2C8

The structure of CYP2C8 active site has been elucidated with pharmacophore, homology and x-ray crystallography models (Melet *et al.* 2004; Schoch *et al.* 2004; Tanaka *et al.* 2004). Based on the crystallography model the volume of the CYP2C8 active site cavity is 1438 Å³. CYP2C8 and CYP3A4 overlap in their substrate specificity; although they only share 25% sequence homology, whereas CYP2C8 and CYP2C9 share only a few substrates despite their 70% sequence homology. This has been attributed to the size or conformation of the active site cavities of CYP2C8 and CYP3A4, which are able to accommodate large drug molecules. The

metabolic products catalysed by CYP2C8 or CYP3A4 are, however, usually different, suggesting that common substrates can bind differently in the active sites of these two enzymes, resulting in for example 6 α -hydroxypaclitaxel catalysed by CYP2C8 and 3'-hydroxypaclitaxel catalysed by CYP3A4 (Totah *et al.* 2005).

In the crystallography model CYP2C8 was originally crystallised as a fatty-acid stabilised dimer, which has been shown to be compatible with substrate and inhibitor binding (Melet *et al.* 2004; Schoch *et al.* 2004; Schoch *et al.* 2008). The substrate binding to CYP2C8 has been further characterised with crystallised complexes of the enzyme with montelukast, troglitazone, felodipine and 9-*cis*-retinoic acid. CYP2C8 exhibited two substrate-binding cavities merging at the proximity of the heme iron of the catalytic site, forming a Y-shaped active site cavity rather large at the junction of the branches. The binding of the studied ligand drugs induced only small local conformational changes in the CYP2C8 protein, and the interactions between the ligand and the protein were predominantly hydrophobic. (Schoch *et al.* 2008)

The ligand-CYP2C8 protein complexes showed full, partial or double occupancy of the active site cavity. The tertiary structure of montelukast with its three branches fitted both the size and shape of the cavity without major changes in the structure of the cavity, size dictating the orientation of the largest branch of montelukast and polarity the orientation of the two other branches, positioning the benzyl ring near the heme iron. Troglitazone, which is more linear than montelukast, filled the upper part of the active site cavity of CYP2C8, leaving an unoccupied residual active-site volume close to the heme. Felodipine is considerably smaller than montelukast, (molecular weight 384), non-linear, and a high affinity inhibitor of CYP2C8 (Walsky *et al.* 2005a; Schoch *et al.* 2008). It was sequestered close to the heme, with water molecules in the remaining portions of the cavity. In contrast to this binding as single drug molecules, two molecules of 9-*cis*-retinoic acid together occupied the three branches of the CYP2C8 active site cavity, in a way where the second molecule of 9-*cis*-retinoic acid was located above the proximal molecule, restraining its position for more efficient oxygenation. (Schoch *et al.* 2008)

7.1.2 CYP2C8 substrates, inhibitors and inducers

CYP2C8 is involved in the biotransformation of over 60 clinically used drugs, that are various in size and structure and represent several therapeutic classes (Lai *et al.* 2009). The prototypic *in vitro* substrate and marker reaction for CYP2C8 is paclitaxel and its 6 α -hydroxylation, with K_m of 5.4–19 μ M. Paclitaxel is metabolised in HLMs primarily by CYP2C8 to 6 α -hydroxypaclitaxel, and to two minor metabolites by CYP3A4 (Harris *et al.* 1994; Rahman *et al.* 1994). Amodiaquine *N*-deethylation is an alternative marker reaction with high affinity and turnover rate.

From drugs in clinical use e.g. oral antidiabetics repaglinide, rosiglitazone and pioglitazone as well as hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase inhibitor cerivastatin are metabolised by CYP2C8 in a significant amount (up to eight, three, four and fivefold increase in AUC, respectively, when administered with concomitant gemfibrozil), with minor contribution by CYP3A4 (Backman *et al.* 2002; Niemi *et al.* 2003a; Niemi *et al.* 2003b; Jaakkola *et al.* 2005). Cerivastatin was withdrawn from the market in 2001 due to increased incidence of rhabdomyolysis, especially when administered with gemfibrozil. Also rosiglitazone and another CYP2C8 substrate, troglitazone, have been withdrawn from the market, rosiglitazone in 2010

due to increased risk of cardiovascular ADRs, and troglitazone in 2000 due to hepatotoxicity (Daily *et al.* 2009).

Repaglinide is a recommended *in vivo* probe for CYP2C8 (EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012), which appears to be the predominant enzyme in its hepatic clearance, whereas CYP3A4 is important during the first-pass metabolism (Hatorp *et al.* 2003; Niemi *et al.* 2003b; Tornio *et al.* 2008a). Repaglinide is also a substrate of OATP1B1, thus having its pharmacokinetics influenced by *SLCO1B1* polymorphism (Niemi *et al.* 2005). As an oral treatment for type II diabetes, it lowers postprandial glucose levels, thereby easily inducing hypoglycemia and requiring blood glucose level monitoring in pharmacokinetic drug interaction studies.

Several CYP2C8 inhibitors have also been recognised, many of which are also substrates of the enzyme. Montelukast is among the most potent inhibitors of CYP2C8 *in vitro* (IC₅₀ 19.6 nM in HLMs), followed by zafirlukast, and also an inhibitor recommended for *in vitro* studies (Walsky *et al.* 2005a; Walsky *et al.* 2005b). *In vivo* neither montelukast nor zafirlukast appear to inhibit CYP2C8, as observed by either of them having no effect on the pharmacokinetics of pioglitazone (Jaakkola *et al.* 2006a), and montelukast not affecting the pharmacokinetics of repaglinide (Kajosaari *et al.* 2006). Other reversible and selective and/or potent inhibitors of CYP2C8 *in vitro* are e.g. trimethoprim, felodipine and candesartan (Wen *et al.* 2002; Walsky *et al.* 2005a).

Mechanism-based inhibitors of CYP2C8 include nortriptyline, isoniazid, amiodarone and verapamil (Lai *et al.* 2009). A well characterised mechanism-based inhibitor of CYP2C8 is the gemfibrozil 1-O-β glucuronide metabolite of gemfibrozil (Ogilvie *et al.* 2006). The inhibitory effect of gemfibrozil on CYP2C8 in human starts rapidly, and reaches its maximum in 1–3 h after ingestion. It abates slowly with full recovery only 96 h after discontinuation of gemfibrozil treatment, the CYP2C8 enzyme turnover half-life being 22 h in humans. The mechanism-based inhibition is also dose-dependent: 50% inhibition can be achieved with a single 30 mg dose and >90% inhibition with a single 900 mg dose or 100 mg twice daily dosing. (Tornio *et al.* 2008a; Backman *et al.* 2009; Honkalammi *et al.* 2011b; Honkalammi *et al.* 2011a; Honkalammi *et al.* 2012b)

CYP2C8 is the most inducible member of the human CYP2C subfamily. It is induced *in vivo* by e.g. rifampicin, phenobarbital and dexamethasone, indicating involvement of at least nuclear receptors PXR, CAR and the glucocorticoid receptor (GR) in its regulation (Chen *et al.* 2009).

7.1.3 CYP2C8 polymorphism

The first effects of CYP2C8 polymorphism were described with paclitaxel and the endogenous substrate arachidonic acid two years after cloning of the CYP2C8 gene. Two new variant alleles were identified: CYP2C8*3 (dual amino acid change Arg139Lys/Lys399Arg) and CYP2C8*2 (Ile269Phe) (Klose *et al.* 1999; Dai *et al.* 2001). Currently, at least 17 CYP2C8 alleles have been identified, and are designated from CYP2C8*1A (wild type) to CYP2C8*14. Six of the alleles have been associated with altered enzyme activity (<http://www.cypalleles.ki.se/>). The most common variant alleles are CYP2C8*2, CYP2C8*3 and CYP2C8*4 (Ile264Met). The allele frequency of CYP2C8*2 is 18% in black population, but it is very rare in Caucasians, whereas CYP2C8*3 is expressed in Caucasians with allele frequency of 23% (Dai *et al.* 2001; Totah *et al.* 2005). The CYP2C8*3 allele is in partial linkage

disequilibrium with the *CYP2C9*2* allele (Yasar *et al.* 2002; Pedersen *et al.* 2010). The allele frequency of *CYP2C8*4* is about 8% in Caucasians (Totah *et al.* 2005).

Several *CYP2C8* haplotypes have also been characterised in different populations (Saito *et al.* 2007; Rodriguez-Antona *et al.* 2008). The strong link between *CYP2C8* and *CYP2C9* genes is seen also in haplotype studies: e.g. a study of subjects from 45 populations worldwide screening for 10 SNPs across *CYP2C8* and *CYP2C9* revealed 17 common haplotypes, and 89% of chromosomes with one of certain two *CYP2C8* variants also carried a certain *CYP2C9* variant allele (Speed *et al.* 2009).

The functional significance of *CYP2C8* polymorphism has been evaluated in several *in vitro* studies, and decreased metabolism of *CYP2C8* substrates by *CYP2C8*2*, *CYP2C8*3* and *CYP2C8*4* alleles have been observed. The clinical pharmacogenetics have also been evaluated for some *CYP2C8* substrates, with results hitherto diverging to some extent from those observed *in vitro*.

In two studies of Caucasian subjects the genotype *CYP2C8*1/*3* was associated with 45% and 48% lower AUC of repaglinide, respectively, compared to wild type homozygotes (Niemi *et al.* 2003c; Niemi *et al.* 2005). When evaluating the effect of polymorphisms of *CYP2C8*, *CYP3A4*, *ABCB1* and *SLCO1B1*, significant predictors for repaglinide AUC were *CYP2C8*1/*3* and *SLCO1B1* genotype (Niemi *et al.* 2005). In two haplotype-phenotype studies haplotype B (causal SNP *CYP2C8*1B*) was associated with increased metabolism of paclitaxel *in vitro*, and in humans, haplotypes B and D (contains *CYP2C8*3* allele) were associated with increased, while haplotype C (when C1, C2 and C3 grouped together) with decreased repaglinide metabolism (Rodriguez-Antona *et al.* 2008; Daily *et al.* 2009). One study found no difference in repaglinide pharmacokinetics between subjects with the *CYP2C8*3* allele and the wild-type (Bidstrup *et al.* 2006). *CYP2C8* genotyping has been performed in a few repaglinide drug interaction studies as well, but the number of subjects with *CYP2C8*1/*3* and *CYP2C8*1/*4* has been too low to draw definitive conclusions (Kajosaari *et al.* 2005; Tornio *et al.* 2008a). Altogether, the *CYP2C8*3* allele has been associated with an increased repaglinide metabolism, but the possible clinical implication of the variability in repaglinide pharmacodynamics resulting from the *CYP2C8* polymorphism in its pharmacokinetics remains yet to be elucidated.

In the case of rosiglitazone or pioglitazone, there are two studies of rosiglitazone and one of pioglitazone suggesting lower plasma concentrations in *CYP2C8*3* allele carriers (Kirchheiner *et al.* 2006; Aquilante *et al.* 2008; Tornio *et al.* 2008b). On the other hand, two studies found no association, but as drug interaction studies by design, they were originally not powered to show effect of genotype on pharmacokinetics (Hruska *et al.* 2005; Pedersen *et al.* 2006). For paclitaxel, ovarian cancer patients who were carriers of *CYP2C8*3* have been shown to have 11% lower clearance of paclitaxel than non-carriers. (Bergmann *et al.* 2011)

Cerivastatin was withdrawn from the market worldwide in 2001 due to myotoxicity, with rhabdomyolysis as its most severe manifestation. This ADR was attributed to increased plasma cerivastatin concentrations resulting from concomitantly administered gemfibrozil, possible genetic variations in *CYP2C8*-mediated metabolism of cerivastatin or transporters involved in its clearance. A case report described three *CYP2C8* polymorphisms in a patient developing rhabdomyolysis after initiating cerivastatin, and the 475delA, resulting in frame shift and protein truncation, was assumed to be the polymorphism causing the decreased *CYP2C8* activity in this patient (Ishikawa *et al.* 2004). When the *CYP2C8* was sequenced for 126 patients, who

had suffered from rhabdomyolysis while receiving cerivastatin, no genetic variation in *CYP2C8* that would indicate higher susceptibility for rhabdomyolysis could be found (Kaspera *et al.* 2010). However, rare inactive frame shift variant V472fsL494 was suggested to increase susceptibility for ADRs. The results also suggested that one or more alleles of *CYP2C8*3* or *CYP2C8*4* could actually lower the risk of e.g. rhabdomyolysis, as human livers carrying these alleles showed increased cerivastatin clearance *in vitro* (Kaspera *et al.* 2010).

7.2 CYP2C9

CYP2C9 complementary DNA was originally cloned in the end of the 1980s and beginning of the 1990s. *CYP2C9* is the predominant form of *CYP2C* enzymes, with largest contribution to human liver microsomal CYP content among the *CYP2C* isoforms and significant amount of substrate drugs. It participates in the biotransformation of over 100 drugs in clinical use, and its substrates are also subject to drug interactions with clinical consequences, especially those with narrow therapeutic range such as phenytoin and warfarin. *CYP2C9* presents significant genetic polymorphism with clinical relevance, when even 40% of Caucasians carry an allele associated with defective functional form of the enzyme (Rettie *et al.* 2005).

The *CYP2C9* gene is located in the human *CYP2C8-CYP2C9-CYP2C19-CYP2C18* gene cluster in the chromosome 10q24, has nine exons, spans about 55kb, and encodes a protein of 490 amino acids (de Morais *et al.* 1993; Gray *et al.* 1995; Wang *et al.* 2009). The enzyme is most abundant in liver, where it accounts for about 20% of the total hepatic CYP content and metabolises about 16% of drugs in clinical use that undergo phase I metabolism (Lewis *et al.* 2002; Rendic 2002; Rettie *et al.* 2005; Pelkonen *et al.* 2008; Wang *et al.* 2009). *CYP2C9* mRNA has also been detected in the kidney, testes, adrenal gland, prostate, ovary and duodenum, and protein has been found in e.g. vascular endothelial cells, where it may together with *CYP2C8* play a role in the metabolism of AA to vasoactive EETs (Enayetallah *et al.* 2004; Rettie *et al.* 2005).

CYP2C9 was the first CYP enzyme that was crystallised, with the absence and presence of its substrate S-warfarin (Williams *et al.* 2003). Warfarin was situated in a predominantly hydrophobic pocket, and its binding to *CYP2C9* induced only small local, but no major conformational changes in the protein. This together with the large active site raises possibility for small molecules to simultaneously bind to the active site. *CYP2C9* has exhibited atypical kinetics, which could result from binding of multiple substrates, and e.g. dapsone has been shown to activate *CYP2C9*-mediated metabolism, supporting a two-site binding model (Hutzler *et al.* 2001). The metabolism of warfarin, however, follows M-M kinetics (Hemeryck *et al.* 1999; Williams *et al.* 2003).

7.2.1 CYP2C9 substrates, inhibitors and inducers

Among the first substrates identified for *CYP2C9* were tolbutamide, phenytoin and S-warfarin (Rettie *et al.* 1992; Zhou *et al.* 2009b). *CYP2C9* also catalyses the metabolism of numerous other clinically relevant drugs: oral sulfonylureas (tolbutamide, glibenclamide, glimepiride), NSAIDs (diclofenac, ibuprofen, ketoprofen, naproxen, indomethacin), cyclo-oxygenases (celecoxib, lumiracoxib, etoricoxib, valdecoxib), antiepileptics (phenytoin, phenobarbital), angiotensin II receptor inhibitors (losartan, irbesartan, candesartan) as well as anticancer drugs

(cyclophosphamide, tamoxifen) and other anticoagulants in addition to S-warfarin (S-acenocumarol, phenprocoumon) (Tang *et al.* 2000; Pelkonen *et al.* 2008; Wang *et al.* 2009). S-warfarin 7-hydroxylation and diclofenac 4'-hydroxylation are validated CYP2C9 marker reactions *in vitro* (Pelkonen *et al.* 2008; EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012). CYP2C9 also participates in the metabolism of several endogenous substrates, such as progesterone, testosterone, 17 α -ethinylestradiol and all-*trans*-retinoic acid (Wang *et al.* 2009).

Known inhibitors of CYP2C9 include amiodarone, fluconazole and voriconazole. Fluconazole is among the recommended inhibitors for CYP2C9 in drug interaction studies (EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012), but unselective, inhibiting also CYP2C19 and CYP3A4 (Olkkola *et al.* 1996; Kaukonen *et al.* 1998; Pelkonen *et al.* 2008). CYP2C9 is induced at least by rifampicin, phenobarbital, phenytoin, carbamazepine and St John's wort, with involvement of CAR and/or PXR receptors (Rendic 2002; Pelkonen *et al.* 2008; Zhou *et al.* 2009b).

7.2.2 CYP2C9 polymorphism

CYP2C9*2 (Arg144Cys) and CYP2C9*3 (Ile359Leu) were the first genetic CYP2C9 polymorphisms recognised. CYP2C9 polymorphism was initially reported in the metabolism of tolbutamide, which was subsequently recognised to result from the CYP2C9*3 allele (Scott *et al.* 1979; Sullivan-Klose *et al.* 1996). The frequency of CYP2C9*2 and CYP2C9*3 variants was 10% and 7.4%, respectively, in Swedish population (Yasar *et al.* 1999). Currently, at least 40 CYP2C9 variant alleles and subvariants have been identified and are designated from CYP2C9*1A (wild type) to CYP2C9*35. (<http://www.cypalleles.ki.se/>). At least six of the CYP2C9 variant alleles have been associated with decreased enzyme function *in vivo*, and several more alleles have been described to decrease the enzyme activity *in vitro* (<http://www.cypalleles.ki.se/>). The first two variant alleles CYP2C9*2 and *3 have allele frequency of 11% and 8% in Caucasians, 3% and 0.8% in Blacks and 0% and 1.6% in Asians, respectively (Xie *et al.* 2001).

Warfarin is used for the treatment and prevention of thrombotic conditions in about 0.5–1.5% of the population, and represents probably the most studied example of the CYP2C9 genotype on the clinical response to its substrate. According to *in vitro* studies, the CYP2C9*2 and *3 variant alleles impair the metabolism of S-warfarin by about 30–40% and 80–90%, respectively. The dosing of warfarin is usually empirical, based on monitoring of the international normalised ratio (INR), with stable doses varying from 1–20 mg/day targeted to result in INR between two and three. The target of warfarin is vitamin K epoxide reductase, encoded by *VKORC1*, which also displays polymorphisms that make patients more sensitive to warfarin. Together the CYP2C9 and *VKORC1* genotypes are the major determinants of warfarin dose, together accounting for almost 50% of the dose variations in patients of European ancestry, being less significant in Asians or Africans. Pharmacogenetic algorithms that guide the dosing of warfarin have been developed, but although the evidence for the association of CYP2C9 and *VKORC1* genotypes in warfarin dosing is robust, the evidence of the clinical benefit of genotyping is still underway. (<http://www.pharmgkb.org/> ; <http://www.warfarindosing.org/> ; Johnson *et al.* 2011; Eriksson *et al.* 2012)

7.3 CYP2C19

CYP2C19 was initially found through the discovery of a PM of mephenytoin, and the subsequent demonstration that S-mephenytoin hydroxylase was CYP2C19 (Wrighton *et al.* 1993; Goldstein *et al.* 1994; Desta *et al.* 2002; Zhou *et al.* 2009a). The *CYP2C19* gene has nine exons, is located in the human *CYP2C8-CYP2C9-CYP2C19-CYP2C18* gene cluster in the chromosome 10q24, and encodes a protein of 490 amino acids (Gray *et al.* 1995; Desta *et al.* 2002). *CYP2C19* is mainly expressed in the liver, where it accounts for about 8% of phase I metabolism (Lewis *et al.* 2002). In extrahepatic tissue it has been detected only in the gut wall in duodenum (Klose *et al.* 1999; Zhou *et al.* 2009a).

7.3.1 CYP2C19 substrates, inhibitors and inducers

CYP2C19 accounts for the metabolism of about 10% of commonly used drugs, including proton pump inhibitors (PPIs; omeprazole, esomeprazole, lansoprazole, pantoprazole), tricyclic antidepressants (imipramine, amitriptyline, nortriptyline), selective serotonin uptake inhibitors (SSRIs; citalopram, fluoxetine, sertraline), benzodiazepines (diazepam, flunitrazepam), and the antiplatelet agent clopidogrel (Desta *et al.* 2002; Pelkonen *et al.* 2008; Zhou *et al.* 2009a).

No selective CYP2C19 inhibitor has been found so far, but e.g. omeprazole, fluconazole and ticlopidine have been used as CYP2C19 inhibitors, all of them having also affinity towards other CYPs (Pelkonen *et al.* 2008). Fluconazole, fluvoxamine and ticlopidine are classified as strong CYP2C19 inhibitors by the FDA guidance, and the EMA guideline lists omeprazole as an example of a moderate CYP2C19 inhibitor (EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012). Omeprazole inhibits also CYP2C9 and CYP3A4 *in vitro*, but has not influenced the pharmacokinetics of S-warfarin *in vivo* (Sutfin *et al.* 1989; Unge *et al.* 1992; Pelkonen *et al.* 2008). Fluvoxamine also inhibits the CYP1A2-mediated metabolism of caffeine in addition to CYP2C19 inhibition on even sub-therapeutic doses (Christensen *et al.* 2002). As for other enzymes in the CYP2C subfamily, CYP2C19 is also induced through the nuclear receptor involvement by e.g. rifampicin, phenytoin, phenobarbital, carbamazepine and St John's wort (Chen *et al.* 2009).

7.3.2 CYP2C19 polymorphism

CYP2C19 also presents polymorphism, and currently, at least 36 *CYP2C19* variant alleles with several subvariants have been identified and designated from *CYP2C19*1A* (wild type) to *CYP2C19*28* (<http://www.cypalleles.ki.se/>). At least 11 of the *CYP2C19* variant alleles have been associated with decreased enzyme function *in vivo*, and one with a gain-of-function (*CYP2C19*17*) (<http://www.cypalleles.ki.se/>). The first variant allele *CYP2C19*2* is a point mutation resulting in splicing defect and truncated non-functional enzyme. Its allele frequency in Caucasians is about 15%, 17% in Blacks and 30% in Chinese. The second common variant allele, *CYP2C19*3*, is a single base transition resulting in premature stop codon and truncated protein. It is rare in Caucasians (0.04%) and Blacks (0.4%) and more frequent in Chinese (5%). *CYP2C19*2* accounts for 75–85% of the *CYP2C19* PM phenotype in Orientals and Caucasians, and together these two alleles are responsible for 90% of the PMs in Caucasians, and for almost all in Blacks and Asian populations (Xie *et al.* 2001; Desta *et al.* 2002). The gain-of-function variant allele *CYP2C19*17* has been observed with an allele frequency of about 20% in Nordic, Greek and African population, and in very low frequencies in Asian

population, varying from 4% in Chinese to 0.3% in Koreans (Pedersen *et al.* 2010). In Nordic populations *CYP2C19*17* also presents strong linkage disequilibrium with the wildtype *CYP2C8*1* and *CYP2C9*1* alleles (Pedersen *et al.* 2010).

Most PPIs are metabolised CYP2C19, and the PM phenotype has been associated with a beneficial effect due to the decreased metabolism of these drugs, leading to an increased drug exposure (5–12 times higher AUC) and an increased healing rate of gastric ulcers (Klotz *et al.* 2004; Klotz 2006). However, there are differences in the effect of *CYP2C19* genotype on the pharmacokinetics of different PPIs, but especially under steady state conditions the contribution of CYP3A4 in their metabolism diminishes the effect of *CYP2C19* genotype (Schwab *et al.* 2005).

CYP2C19 polymorphism and its association with the clinical response of clopidogrel has been widely studied and debated. Clopidogrel is an antiplatelet drug used by 40 million patients worldwide, and its platelet inhibitory effect response is heterogeneous. It is a prodrug, and transformed to its active metabolite by pathways involving CYP enzymes, CYP2C19 thought to be the most important in its activation. In 2009 the FDA recommended *CYP2C19* genotyping prior to prescription of clopidogrel (Holmes *et al.* 2011). The *CYP2C19* genotype with the gain-of-function allele has been associated with increased risk for bleeding complications but no significant effect on the occurrence of stent thrombosis (Sibbing *et al.* 2010). The *CYP2C19* genotypes with loss-of-function allele had been associated with about 1.5–2 times higher risk of occurrence of major adverse cardiovascular event or cardiovascular death and even four times higher risk of stent thrombosis in several systematic reviews (Montalescot *et al.* 2009; Hulot *et al.* 2010; Mega *et al.* 2010; Jin *et al.* 2011; Sofi *et al.* 2011). In genome-wide association studies the *CYP2C19*2* variant accounted for about 12% of the overall variation of clopidogrel platelet response (Shuldiner *et al.* 2009; Topol *et al.* 2011). More recent meta-analyses have shown an association between the *CYP2C19* genotype and clopidogrel responsiveness, but no significant association of the genotype with cardiovascular events, or even no substantial influence of the a *CYP2C19* genotype on the clinical efficacy of clopidogrel (Bauer *et al.* 2011; Holmes *et al.* 2011).

7.4 CYP3A4

CYP3A4 is the most abundant member of the CYP3A subfamily in humans. It is the major hepatic CYP isoform expressed in adults, constituting about 30% of the total hepatic CYP content, and performs 35% of the CYP-mediated drug oxidations (Thummel *et al.* 1998; Daly 2004). CYP3A4 is also present in about 50% of its hepatic levels in the small intestine epithelium, accounting for about 70% of total CYP content in the small intestine, displaying, however, even tenfold interindividual heterogeneity in its expression (Lown *et al.* 1994; Shimada *et al.* 1994; Paine *et al.* 1997). As CYP3A4 shares many substrates with P-gp, their coordinated action in the small intestine contributes significantly to reduced bioavailability of orally administered drugs. (Paine *et al.* 1997; Thummel *et al.* 1998; Koch *et al.* 2002; Bachmann *et al.* 2005)

The human *CYP3A4* gene is located in the *CYP3A* locus on chromosome 7q21.1–22.1, consists of 13 exons and spans 27 kb (Lamba *et al.* 2006; Zhou *et al.* 2009a). Its crystal structure indicates an active site of 520 Å³ in volume, which is smaller than anticipated based on its substrate size. The heme of CYP3A4 was more accessible to the active site than for

example that of CYP2C9, and indicated that CYP3A4 is able to bind and metabolize multiple substrates simultaneously (Williams *et al.* 2004).

Currently at least 40 *CYP3A4* variant alleles with several subvariants have been identified and designated from *CYP3A4*1A* (wild type) to *CYP3A4*22*, as well as several SNPs where the haplotype has not been determined yet (<http://www.cypalleles.ki.se/>). Two of the variant alleles have been associated with an altered enzyme function, but otherwise *CYP3A4* does not exhibit clinically relevant polymorphism. The *CYP3A4*22* variant allele has been associated with reduced CYP3A4 activity *in vivo*, and need for lower statin doses and better lipid lowering response to simvastatin, as well as higher tacrolimus and cyclosporine concentrations, especially when combined with *CYP3A5*3* SNPs (Elens *et al.* 2011a; Elens *et al.* 2011b; Wang *et al.* 2011). Variant allele *CYP3A4*18A* has been associated with low bone mass, suggested to result from a gain-of-function mutation in sex steroid metabolism (Kang *et al.* 2009).

7.4.1 CYP3A4 substrates, inhibitors and inducers

CYP3A4 is estimated to participate in the metabolism of about 50% of all drugs in clinical use (Bachmann *et al.* 2005). The numerous substrates of CYP3A4 include cyclosporine, erythromycin, clarithromycin, midazolam, triazolam, simvastatin and atorvastatin, as well as endogenous substrates such as testosterone, progesterone and bile acids (Pelkonen *et al.* 2008). Midazolam 1-hydroxylation and testosterone 6 β -hydroxylation are validated *in vitro* marker reactions, and several substrates can be used in *in vivo* studies, oral midazolam being recommended for classification of the inhibitor potency (EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012). Several of the CYP3A4 substrates are also substrates of CYP2C8, P-gp and/or OATB1B1, such as simvastatin, lovastatin, atorvastatin, fluvastatin and cerivastatin (Neuvonen *et al.* 2006).

As CYP3A4 has a low substrate specificity, it is also susceptible to several structurally different inhibitors. Itraconazole is a highly selective competitive inhibitor of CYP3A4, resulting in e.g. 8-fold increase of the AUC of midazolam (Backman *et al.* 1998). Verapamil, diltiazem and saquinavir are also selective, mechanism-based inhibitors of CYP3A4, and verapamil and diltiazem have resulted in three- and fourfold increase in the AUC of midazolam (Backman *et al.* 1994; Pelkonen *et al.* 2008). The most potent inducers of CYP3A4 are rifampicin and rifabutin, resulting in up to 40-fold reduction in their substrate plasma concentrations. Also carbamazepine, phenytoin and phenobarbital induce CYP3A4 (Backman *et al.* 1996a; Backman *et al.* 1996b; Backman *et al.* 1998; Pelkonen *et al.* 2008). As for CYP3A4 substrates, overlap occurs also with inhibitors of CYP2C8, P-gp and OATP1B1.

7.4.2 Other members of the CYP3A subfamily

Three other members of the CYP3A subfamily have been isolated in humans. CYP3A7 was originally found in the human fetal liver, being the dominant CYP3A enzyme in embryonic, fetal and neonate liver, but down-regulated after birth and during the first year of life, although being present also in the adult liver and the small intestine (Schuetz *et al.* 1994; Lacroix *et al.* 1997; Koch *et al.* 2002; Fakhoury *et al.* 2005). The expression and function of CYP3A43 is the least known of CYP3A subfamily members, though it has been found in the liver, prostate and testis (Daly 2006).

CYP3A5 is present in the liver, intestine, kidney, adrenal gland and prostate (Wrighton *et al.* 1990; Schuetz *et al.* 1994; Koch *et al.* 2002). The CYP3A5*3 allele produces an incorrectly spliced mRNA resulting in truncated, non-functional enzyme, and the expression of CYP3A5 is determined by the CYP3A5 genotype. CYP3A5 accounts for 50% of the total hepatic CYP3A content in individuals with at least one CYP3A5*1 (wild-type) allele, for 76% in those homozygous for the CYP3A5*1 allele, and only 4% for those homozygous for the CYP3A5*3 allele. 82–86% of Whites are homozygous for the CYP3A5*3 allele, i.e. so-called CYP3A5 non-expressors. (Barry *et al.* 2010)

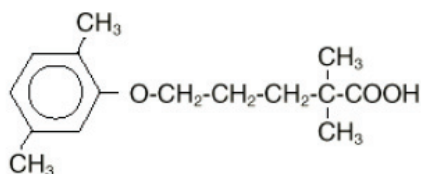
7.5 CYP inhibitor drugs

7.5.1 Gemfibrozil

Gemfibrozil is a lipid lowering drug that was introduced to the market in 1976 (Betteridge *et al.* 1976). The effect of gemfibrozil in the primary prevention of hyperlipidaemia patients has been demonstrated in e.g. the Helsinki Heart study, where it reduced the plasma triglyceride level by 35% and cholesterol level by 11% with an 11% increase on high-density lipoprotein (HDL) (Frick *et al.* 1987). The mechanism of action of gemfibrozil has not been totally unravelled, but nuclear hormone receptors of PPAR- α -type are suggested to be its target proteins. Activation of PPAR- α has several effects, e.g. stimulation of endosomal mobilisation of cholesterol towards the plasma membrane, increase in the catabolism of very long chain fatty acids of low density lipoprotein (LDL) and very low density lipoprotein (VLDL), as well as stimulation of the production of HDL (Roy *et al.* 2009a).

The bioavailability of gemfibrozil after oral administration is 100%, and it is 98% bound to plasma proteins. The t_{\max} is reached in 1–2 h, and $t_{1/2}$ is about 1.5 h. The main route of elimination is renal, i.e. 70% is excreted in the urine, mostly as gemfibrozil 1-O- β glucuronide and less than 2% as unchanged gemfibrozil. Gemfibrozil also undergoes oxidation resulting in hydroxymethyl and carboxymethyl metabolites. (Miller *et al.* 1998; Lopid Label 2010)

Figure 7.5.1.1 Chemical structure of gemfibrozil (Lopid Label 2010)



In vitro gemfibrozil is a CYP2C9, CYP2C19, CYP2C8 and CYP1A2 inhibitor, with the lowest IC_{50} for CYP2C9 (30 μ M), and thereafter for CYP1A2, CYP2C19 and CYP2C8 (99, 100 and 120, respectively) (Wen *et al.* 2001; Ogilvie *et al.* 2006). The main metabolite, gemfibrozil 1-O- β glucuronide is less effective inhibitor of all other CYP enzymes than gemfibrozil, except for CYP2C8. The acyl glucuronidation of gemfibrozil results in gemfibrozil 1-O- β glucuronide,

which is a mechanism-based inhibitor of CYP2C8. It potently and selectively inhibits CYP2C8 with an order of magnitude lower IC_{50} (24 μ M) than the other CYPs, and with pre-incubation the inhibition increases even an order of magnitude more (IC_{50} 1.8 μ M) (Ogilvie *et al.* 2006).

The inhibitory effect of gemfibrozil on CYP2C8 observed *in vivo* is thought to result from this mechanism-based inhibition of CYP2C8 by gemfibrozil 1-O- β glucuronide (Shitara *et al.* 2004; Ogilvie *et al.* 2006; Baer *et al.* 2009). Gemfibrozil results in total inactivation of the CYP2C8 enzyme, which starts rapidly within an hour, and is only overcome with *de novo* synthesis of the enzyme in 2–3 days (Backman *et al.* 2009; Honkalammi *et al.* 2011b). It is listed as a strong CYP2C8 inhibitor in both the FDA and EMA guidelines (Huang *et al.* 2007; EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012).

The effect of gemfibrozil on the pharmacokinetics of numerous clinically used CYP2C8 substrates has been demonstrated, including cerivastatin, simvastatin acid, pravastatin, repaglinide, pioglitazone, rosiglitazone and loperamide (Backman *et al.* 2000a; Backman *et al.* 2002; Kyrklund *et al.* 2003; Niemi *et al.* 2003b; Jaakkola *et al.* 2005). Combination therapy of gemfibrozil with repaglinide is contraindicated due the risk of severe hypoglycaemia, and concomitant treatment with HMG-CoA reductase inhibitors warrants precaution due to increased risk of myopathy and rhabdomyolysis (Lopid Label 2010). The adverse effects of HMG-CoA reductase inhibitors administered concomitantly with gemfibrozil are linked also to the inhibition of the transporter OATP1B1 by gemfibrozil and gemfibrozil 1-O- β glucuronide (Shitara *et al.* 2004; Neuvonen *et al.* 2006). This interferes with the entry of HMG-CoA reductase inhibitors to the hepatocytes, their site of action, as well as their elimination.

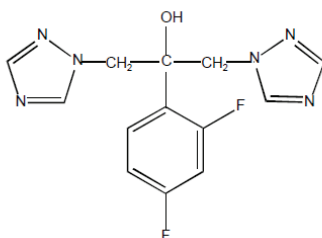
Although gemfibrozil potently inhibits CYP2C9 *in vitro*, it had only limited effects on the pharmacodynamics of warfarin in healthy volunteers: the AUC of S-warfarin decreased by 11% by gemfibrozil (Lilja *et al.* 2005). There are some case reports of patients receiving warfarin experiencing increase in their INR values after initiation of gemfibrozil, and thus requiring a reduction in the dose of warfarin (Dixon *et al.* 2009). The mechanism of this phenomenon may involve displacement of warfarin from plasma albumin or interference in the absorption of warfarin (Lilja *et al.* 2005; Dixon *et al.* 2009).

7.5.2 Fluconazole

Fluconazole is an antifungal agent of the triazole group and acts by inhibiting the fungal sterol synthesis. It is used for the treatment of several fungal infections varying from dermal fungal infections and genital and mucous membrane candidiasis to systemic candidiasis and Cryptococci infections.

Fluconazole is available both as peroral and intravenous formulations. Its bioavailability after oral administration is 90%. The t_{max} is reached in 1–2 h, binding to plasma proteins is 11–12%, and $t_{1/2}$ is about 30 h. Fluconazole reaches high concentrations (80% of plasma concentrations) in the cerebrospinal fluid, and fungicidal concentrations also in e.g. the vagina, saliva, skin and nails, with the ratio of fluconazole tissue/plasma concentration ranging from 0.4–0.7 in vaginal fluid up to 10 in skin. Fluconazole is cleared primarily by renal excretion so that 80% is excreted unchanged in the urine, and 11% as metabolites. Impaired renal function directly affects fluconazole pharmacokinetics, warranting dose reduction. (Rang *et al.* 2007a; Diflucan Label 2011)

Figure 7.5.2.1 Chemical structure of fluconazole (Diflucan Label 2011)



Fluconazole is a non-competitive inhibitor of CYP2C9, CYP2C19 and CYP3A4, and interacts directly with the heme moiety of these enzymes (Nivoix *et al.* 2008). *In vitro*, the fluconazole K_i for CYP2C9-mediated metabolism of S-warfarin is 7–8 μM , for CYP3A4-mediated metabolism of R-warfarin 15–18 μM , and about 9 μM for CYP2C19-mediated hydroxylation of R-warfarin (Kunze *et al.* 1996; Wienkers *et al.* 1996).

In vivo the inhibitory effect of fluconazole on CYP2C9, CYP2C19 and CYP3A4 has been demonstrated in numerous drug interaction studies, showing a strong CYP2C9 and moderate CYP3A4 inhibitory effect (Nivoix *et al.* 2008; Diflucan Label 2011). The fluconazole IC_{50} against CYP2C9, CYP2C19 and CYP3A4 is 30, 12 and 13 μM , respectively (Niwa *et al.* 2005). Fluconazole 400 mg daily inhibited the CYP2C9-mediated hydroxylations of S-warfarin by 70% and the CYP3A4-mediated hydroxylation of R-warfarin by 45%, causing two- and threefold increase in the AUC of S- and R-warfarin (Black *et al.* 1996). The CYP3A4 inhibitory effect of fluconazole is dose dependent: 100 mg fluconazole daily increased the AUC of triazolam 2.1-fold, whereas 400 mg daily increased the AUC of triazolam 4.4.-fold (Varhe *et al.* 1996). Fluconazole has also increased the AUC of midazolam 3.6-fold, diazepam 2.5-fold and omeprazole 6.3-fold (Oikkola *et al.* 1996; Kang *et al.* 2002; Saari *et al.* 2007), and can thus produce significant interactions with other CYP2C9, CYP2C19 and CYP3A4 substrates as well. In addition to CYP inhibition, fluconazole interacts with phase II enzymes involved in glucuronidation, and has inhibited e.g. the glucuronidation of zidovudine (Kiang *et al.* 2005; Nivoix *et al.* 2008).

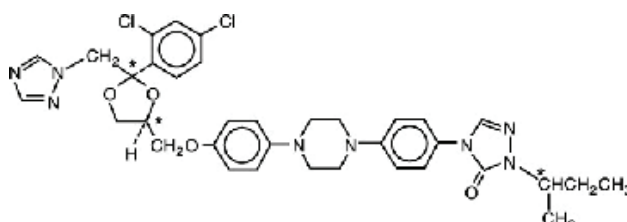
7.5.3 Itraconazole

Itraconazole is another triazole antifungal drug that acts by inhibiting the fungal CYP enzyme mediating lanosterol 14 α -demethylation, thus interfering with the fungal cell membrane ergosterol synthesis. It is active against several dermatophytes, but does not penetrate the cerebrospinal fluid. It is used in the treatment of gynaecological and dermal fungal infections, as well as systemic fungal infections, including Cryptococci infections outside the brain.

The bioavailability of itraconazole after oral administration is about 55%. Its absorption is impaired in situations where the gastric pH increases, and its bioavailability is maximal after a full meal. The t_{max} is about 3–5 h, and plasma protein binding 99.8 %. Itraconazole is lipophilic, and the volume of distribution is large, 11L/kg. Itraconazole accumulates in tissues, including

the skin, nails and female genitals; with the highest ratio of itraconazole tissue/plasma concentration in skin (10) and fat (17). Itraconazole is metabolised predominantly by CYP3A4, and hydroxyitraconazole (OH-itraconazole) is the major metabolite. Faecal excretion of the parent itraconazole is 3–18% of the dose, and renal excretion is minimal (less than 0.03% of the dose). About 40% of the dose is, however, excreted in the urine as inactive metabolites. Its $t_{1/2}$ after single dose is about 21 h and increases to 30–60 h in steady-state concentrations, indicating saturation of its metabolism. The active metabolites OH-itraconazole and *N*-desalkyl-itraconazole are thought to contribute to the saturation of CYP3A4. (De Beule *et al.* 2001; Willems *et al.* 2001; Rang *et al.* 2007a; Nivoix *et al.* 2008; Sporanox Label 2012)

Figure 7.5.3.1 Chemical structure of itraconazole (Sporanox Label 2012)



Itraconazole is a strong inhibitor of CYP3A4, and also listed as such in both the FDA and EMA guidelines (EMA guideline on the investigation of Drug Interactions (draft) 2010; EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012). It has resulted in significant increases in the AUCs of several CYP3A4 substrates, including midazolam, felodipine, buspirone and lovastatin (Olkola *et al.* 1994; Neuvonen *et al.* 1996; Olkkola *et al.* 1996; Jalava *et al.* 1997a; Kivistö *et al.* 1997). Itraconazole resulted in drastic, 27-fold increase in triazolam AUC (Varhe *et al.* 1994), and the inhibitory effect is strong even four days after cessation of itraconazole (Backman *et al.* 1998). Itraconazole has not affected the pharmacokinetics of CYP2C9 substrate losartan (Kaukonen *et al.* 1998).

Being a substrate of CYP3A4, itraconazole pharmacokinetics are affected by other CYP3A4 inhibitors. The metabolism of itraconazole is also affected by CYP3A4 inducers, and may lead to clinical consequences due to sub-therapeutic plasma concentrations. Phenytoin, for example has resulted in tenfold reduction of itraconazole concentrations (Ducharme *et al.* 1995), and rifampicin in 88% decrease of the itraconazole AUC (Jaruratanasirikul *et al.* 1998).

In drug interaction studies where itraconazole and gemfibrozil have been used as probe inhibitors, about 50% lower plasma concentrations of itraconazole and OH-itraconazole have been observed when itraconazole and gemfibrozil were administered concomitantly, compared to itraconazole alone. This has been discussed to result e.g. from reduced bioavailability of itraconazole or its displacement from plasma proteins by gemfibrozil (Niemi *et al.* 2003b; Jaakkola *et al.* 2005).

AIMS OF THE STUDY

The aim of this thesis was to study the roles of CYP enzymes in the *in vivo* metabolism of montelukast and zafirlukast in humans by using probe inhibitors.

The specific aims of the studies were:

- I To investigate the effects of CYP2C8 inhibitor gemfibrozil on the pharmacokinetics of montelukast in healthy subjects. In addition, to investigate the effects of gemfibrozil and its 1-O- β glucuronide on montelukast metabolism *in vitro*.
- II To investigate the effects of CYP3A4 inhibitor itraconazole, CYP2C8 inhibitor gemfibrozil and their combination on the pharmacokinetics of montelukast in healthy subjects.
- III To investigate the effects of CYP2C8 inhibitor gemfibrozil on the pharmacokinetics of zafirlukast in healthy subjects.
- IV To investigate the effects of CYP3A4 inhibitor itraconazole and CYP2C9 and CYP3A4 inhibitor fluconazole on the pharmacokinetics of zafirlukast in healthy subjects.

MATERIALS AND METHODS

1 *In vitro* study (Study I)

To study the effect of gemfibrozil on montelukast or M6 metabolism, montelukast or M6 and various concentrations of gemfibrozil were premixed for three minutes with HLMS and buffer. The reaction was started by adding β -NADPH.

To study the effect of gemfibrozil 1-O- β glucuronide on montelukast or M6 metabolism, it was first premixed for three minutes with microsomes and buffer. After addition of β -NADPH, the solution was preincubated for 30 minutes. After this, an aliquot of the mixture was moved to another tube containing β -NADPH, MgCl_2 and phosphate buffer, resulting in a 40-fold dilution. The reaction was immediately started by adding montelukast or M6.

Incubations were terminated at 15 minutes (montelukast) or 30 minutes (M6). The incubation time was in the linear range for the rate of metabolite formation. After vortexing and centrifuging the samples they were subject to analysis of M6 concentrations, or metabolite/internal standard (IS) peak height ratio (M4 and M5b). The IC_{50} of gemfibrozil and gemfibrozil 1-O- β glucuronide was determined by non-linear regression analysis.

2 *In vivo* studies (Studies I-IV)

2.1 Subjects

A total of 42 healthy volunteers participated in the studies (Table 2.1.1). The subjects were ascertained to be healthy by medical history, clinical examination, and routine laboratory tests. None received continuous medication, used hormonal contraception or was a tobacco smoker. Female subjects gave a negative pregnancy test before entering the studies. The routine laboratory tests were not repeated during the study. The use of grapefruit juice or any pharmaceuticals was prohibited a week prior to, during, and 0–7 days after the study, depending on the drugs being studied. Participation in any other study and blood donation was also prohibited three months prior to and after the studies (inclusion and exclusion criteria in Table 2.1.2).

Table 2.1.1 Characteristics of the subjects

| Study | Subjects (female/male) | Age (y) | Height (cm) | Weight (kg) | BMI (kg/m^2) |
|-------|---------------------------|--------------|---------------|-------------|--------------------------------|
| I | 10 (4/6) | 23 ± 1.7 | 173 ± 8.1 | 69 ± 11 | 23 ± 3.0 |
| II | 11 (3/8) | 23 ± 2.0 | 183 ± 6.2 | 78 ± 13 | 23 ± 2.7 |
| III | 9 (3/6) | 22 ± 2.4 | 177 ± 7.3 | 72 ± 11 | 23 ± 2.3 |
| IV | 12 (4/8) | 22 ± 2.6 | 171 ± 11 | 72 ± 12 | 24 ± 2.6 |

Age, weight and body mass index (BMI) data are mean \pm SD (standard deviation)

Table 2.1.2 Inclusion and exclusion criteria in studies I–IV

| Inclusion criteria | | Exclusion criteria | |
|--------------------|--|--------------------|---|
| 1. | Informed consent | 1. | Significant medical condition/illness |
| 2. | Age 18–40 years | 2. | Smoking |
| 3. | Healthy as ascertained by medical history, clinical examination and routine laboratory test | 3. | Hormonal contraception or other continuous medication |
| 4. | Acceptable results in laboratory test: - Hb within reference range - B-PVK, P-ALAT, P-AFOS, P-GT, P-Krea, P-K and P-Na not clinically significantly diverging from the reference range | 4. | Pregnancy or planning of pregnancy |
| 5. | Negative pregnancy test (S-hCG) | 5. | Lactation |
| | | 6. | <3 months since participating a clinical study |
| | | 7. | <3 months since donating blood |
| | | 8. | Significant overweight or poor forearm veins |
| | | 9. | BMI < 18.5 kg/m ² |
| Hb | haemoglobin | | |
| B-PVK | basic blood count | | |
| P-ALAT | plasma alanine aminotransferase | | |
| P-AFOS | plasma alkaline phosphatase | | |
| P-GT | plasma glutamyl transferase | | |
| P-Krea | plasma creatinine | | |
| P-K | plasma potassium | | |
| P-Na | plasma sodium | | |
| S-hCG | serum human chorionic gonadotropin | | |

2.2 Study design

The studies were carried out at the Department of Clinical Pharmacology, University of Helsinki. All the four studies had a randomised, placebo-controlled cross-over design (Table 2.2.1). The studies consisted of two to four phases, and each phase included a pre-treatment period with the inhibitor drug studied or placebo, which was followed by the ingestion of a single oral dose of the study drug on the study day.

The pre-treatments, placebos and the study drugs were supplied, packed and labelled according to a randomisation list for each subject by the Helsinki University Central Hospital Pharmacy.

After an overnight fast, the study drugs were administered orally with 150 ml water at 09:00 on the study day. The subjects received a standardised warm meal three hours, and light meal seven and 10 hours after the study drug intake. During the study days the subjects were under medical supervision for 12 hours after study drug administration.

Table 2.2.1 Study designs

| Study | Pre-treatment | Pre-treatment duration (days) | Wash-out period (weeks) | Study drug |
|-------|--|-------------------------------|-------------------------|-------------------------------------|
| I | 1. Gemfibrozil 600 mg x 2 2. Placebo x 2 | 3 | 4 | Montelukast 10 mg on day 3 at 09:00 |
| II | 1. Gemfibrozil 600 mg x 2 2. Itraconazole 100 mg x2 (first dose 200 mg) 3. Gemfibrozil+itraconazole x2 4. Placebo x 2 | 5 | 4 | Montelukast 10 mg on day 3 at 09:00 |
| III | 1. Gemfibrozil 600 mg x 2 2. Placebo x 2 | 5 | 4 | Zafirlukast 20 mg on day 3 at 09:00 |
| IV | 1. Fluconazole 200mg x 1 (first dose 400mg) 2. Itraconazole 100 mg x2 (first dose 200 mg) 3. Placebo x 2 | 5 | 4 | Zafirlukast 20 mg on day 3 at 09:00 |

2.3 Blood sampling and determination of plasma drug concentrations

On the days of the study drug administrations, a forearm vein of each subject was cannulated for blood sampling. Timed blood samples (5 or 10 ml each in studies I and III, 4 or 9 ml in studies II and IV) were collected into tubes containing ethylenediaminetetraacetic acid (EDTA). Plasma was separated immediately and stored at -70°C until analysis.

The plasma concentrations of montelukast, its metabolites and zafirlukast, as well as gemfibrozil and gemfibrozil 1-O- β glucuronide were determined using PE SCIEX liquid chromatography-tandem mass spectrometry (LC/MS/MS) methods. When no authentic reference compound was available, the concentrations of the metabolites were measured as arbitrary units (AU/ml) based on the ratio of the peak height of each metabolite to that of the IS in the chromatogram (Table 2.3.1). The plasma concentrations of fluconazole, itraconazole and OH-itraconazole were determined by high performance liquid chromatography (HPLC).

Table 2.3.1. Performance of the plasma drug concentration determinations in studies I–IV

| Study | Analytes | Limit of quantification | Interday CV ¹ |
|-------|--|-------------------------|--------------------------|
| I | Montelukast | 2 ng/ml | ≤17% |
| | Montelukast metabolite M6 | 2 ng/ml | ≤10% |
| | Montelukast metabolite M5 ² | s/n ≥10 | |
| | Montelukast metabolite M4 ² | s/n ≥10 | |
| | Gemfibrozil | 2.5 ng/ml (0.0025 mg/l) | ≤4% |
| | Gemfibrozil 1-O-β glucuronide | 2.5 ng/ml (0.0025 mg/l) | ≤8% |
| II | Montelukast | 0.3 ng/ml | <8% |
| | Montelukast metabolite M6 | 1 ng/ml | ≤14% |
| | Montelukast metabolite M5 ² | s/n ≥10 | |
| | Montelukast metabolite M4 ² | s/n ≥10 | |
| | Gemfibrozil | 100 ng/ml (0.1 mg/l) | ≤6% |
| | Gemfibrozil 1-O-β glucuronide | 50 ng/ml (0.05 mg/l) | ≤10% |
| | Itraconazole | 20 ng/ml | ≤3% |
| | OH-itraconazole | 20 ng/ml | ≤2% |
| III | Zafirlukast | 0.5 ng/ml | ≤11% |
| | Gemfibrozil | 2.5 ng/ml (0.0025 mg/l) | <5% |
| | Gemfibrozil 1-O-β glucuronide | 2.5 ng/ml (0.0025 mg/l) | ≤8% |
| IV | Zafirlukast | 0.2 ng/ml | ≤10% |
| | Fluconazole | 200 ng/ml (0.2 mg/l) | <2% |
| | Itraconazole | 10 ng/ml | <12% |
| | OH-itraconazole | 10 ng/ml | ≤9% |

1 Highest interday coefficient for variation (CV) for the assay at relevant concentrations

2 No reference compound available for the metabolite

s/n signal-to-noise ratio

2.4 Pharmacokinetic calculations

The pharmacokinetics of the study drugs, pre-treatments and their metabolites were characterised by C_{\max} , t_{\max} , $t_{1/2}$, and AUC from zero to the last point of measurement (AUC_{0-t}) or infinity (AUC_{∞}). The C_{\max} and t_{\max} values were taken directly from the original data. The terminal log-linear part of each plasma concentration-time curve was identified visually, and the elimination rate constant (k_e) was determined by linear regression analysis of the log-linear part of the concentration-time curve. The $t_{1/2}$ was calculated by the equation $t_{1/2} = \ln 2/k_e$. The AUC values were calculated by the linear trapezoidal rule for the rising phase and the log-linear trapezoidal rule for the descending phase, with extrapolation to infinity by division of the last measured concentration by k_e . The pharmacokinetic calculations were performed with MK-Model, version 5.0 (Biosoft, Cambridge, UK).

2.5 Genotyping

For genotyping in studies I and IV, an EDTA blood sample was drawn from each subject and stored at -70°C/-20°C prior to genomic deoxyribonucleic acid (DNA) extraction with standard methods (Qiaamp DNA Blood Mini Kit, Qiagen, Hilden, Germany). In study I the subjects were genotyped for the *CYP2C8**3 (c.416G>A and c.1196A>G), *CYP2C8**4 (c.792C>G) and *CYP3A5**3 (g.6986A>G) alleles by allelic discrimination with TaqMan® 5'-nuclease assays on an Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). In study IV the subjects were genotyped for the *CYP2C9**2 (c.430C>T, p.R144C; rs1799853), *CYP2C9**3 (c.1075A>C, p.I159L; rs1057910), *CYP2C19**2 (c.681G>A, splicing defect; rs4244285) and *CYP2C19**3 (c.636G>A, p.W212X; rs4986893) loss-of-function alleles and the *CYP2C19**17 (g.-806C>T; rs12248560) gain-of-function allele with TaqMan® Drug Metabolism genotyping assays on an Applied Biosystems 7300 Real-Time PCR system.

2.6 Statistical analysis

In all studies the level of statistical significance was $P < 0.05$ and all the statistical analyses were performed with SPSS for Windows version 17.0 (SPSS Inc, Chicago, IL, USA).

In studies I and III, the results were expressed as a mean \pm standard deviation (SD), except for t_{\max} , which was expressed as a median (range). The pharmacokinetic variables between the two phases were compared with the t-test, or, in the case of t_{\max} , with the Wilcoxon signed-rank test. In study III, logarithmic transformation of C_{\max} and AUC values was performed before statistical analysis, and 95% confidence intervals (CI) were calculated for the geometric mean ratios of these variables.

In study II, the pharmacokinetic variables for montelukast and metabolite M6 were expressed as a geometric mean and coefficient of variation (CV) in the text and table, except for t_{\max} . Because some of the metabolite concentrations were below their lower limit of quantification (LLQ) in some of the study phases, the results for metabolites M4, M5a and M5b were expressed as a median and range in the text. For clarity, all concentrations were given as mean values \pm standard error (SEM) in the figures. For montelukast and metabolite M6 all pharmacokinetic variables, except t_{\max} , were transformed with natural logarithm and the statistical comparisons between the four phases were performed with a repeated-measures ANOVA, followed by a posteriori testing with the paired t-test with the Bonferroni correction. All t_{\max} values and all pharmacokinetic variables of M4, M5a and M5b were compared with the Friedman 2-way ANOVA followed by the Wilcoxon signed rank test with the Bonferroni correction.

In study IV, the results were expressed as geometric mean and CV or 90% CIs, except for t_{\max} , which was expressed as a median (range). All pharmacokinetic variables, except t_{\max} , were transformed with natural logarithm and the statistical comparisons between the three phases were performed with a repeated-measures ANOVA, followed by a posteriori testing with the paired t-test with the Bonferroni correction. Corresponding 95% CIs were calculated for the ratio to control. All t_{\max} values were compared with the Friedman 2-way ANOVA followed by the Wilcoxon signed rank test with the Bonferroni correction. The body weight standardised (to 70 kg) zafirlukast AUC_∞ values between the *CYP2C9* and *CYP2C19* genotypes were compared after logarithmic transformation with ANOVA followed by the Tukey test.

RESULTS

1 Pharmacokinetics of montelukast and its metabolites (Studies I and II)

1.1 Effects of gemfibrozil and gemfibrozil 1-O- β glucuronide *in vitro* (Study I)

Gemfibrozil 1-O- β glucuronide was over 1000-fold more potent than gemfibrozil as an inhibitor of the formation of M6 from montelukast in HLMS. With a 30 min preincubation, gemfibrozil 1-O- β glucuronide inhibited M6 formation with an IC_{50} of 0.074 μ M (0.032 mg/l) and the further metabolism of M6 to M4 with an IC_{50} of 0.11 μ M (0.048 mg/l). Gemfibrozil, in turn, inhibited the formation of M6 with an IC_{50} of 107 μ M (27 mg/l) and the formation of M4 from M6 with an IC_{50} of 63 μ M (16 mg/l). Neither gemfibrozil nor gemfibrozil 1-O- β glucuronide had an effect on the formation of M5b from montelukast.

1.2 Effects of gemfibrozil *in vivo*

Montelukast: In study I, gemfibrozil, compared to the placebo, raised the mean AUC_{∞} of montelukast 4.5-fold ($P < 0.001$), and mean C_{max} 1.5-fold ($P < 0.001$), and prolonged its $t_{1/2}$ 3.0-fold, from 4.5 h to 13.5 h ($P < 0.001$) (Figure 1.2.1).

In study II, the effects of gemfibrozil on montelukast pharmacokinetics were in line with those observed in study I (Figure 1.2.1). During the gemfibrozil phase, the geometric mean ratio to control of the AUC_{∞} of montelukast was 4.3-fold (95% CI 2.9–6.3, $P < 0.001$), and that of its $t_{1/2}$ was 2.1-fold (95% CI 1.7–2.5, $P < 0.001$), i.e. the $t_{1/2}$ was prolonged from 6.4 h to 13 h.

M6: In study I, the formation rate of the primary metabolite M6 was reduced by gemfibrozil: compared to the placebo, gemfibrozil prolonged the median t_{max} of M6 threefold and reduced its mean AUC_{0-7} to 60% of control. The AUC_{0-24} of M6 was increased by 86% ($P = 0.005$) by gemfibrozil.

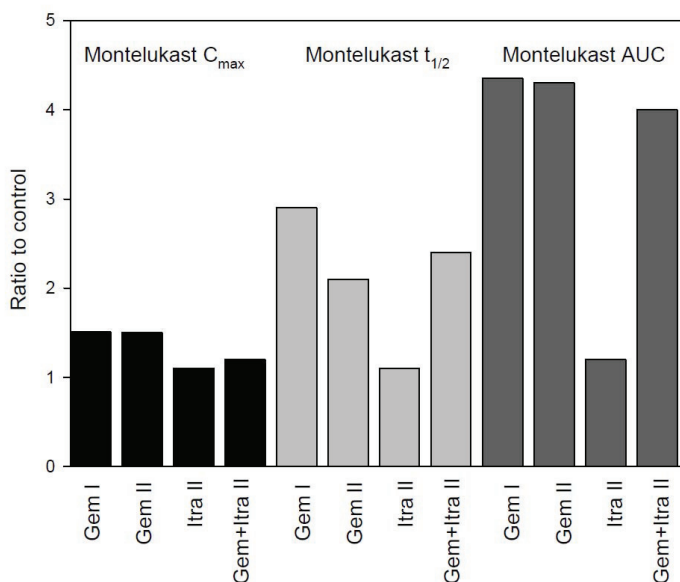
Similarly, in study II, gemfibrozil reduced the formation rate of the metabolite M6. The AUC_{∞} of M6 was increased 3.5-fold (95% CI 2.4–5.0, $P < 0.001$) by gemfibrozil. The $t_{1/2}$ of M6 was prolonged 4.1-fold (95% CI 3.4–4.8, $P < 0.001$) by gemfibrozil.

M4: In study I gemfibrozil clearly reduced the plasma concentrations of the secondary metabolite M4: the C_{max} of M4 was reduced to 9.0% of control ($P < 0.001$) and its AUC_{0-24} to about 10% of control ($P < 0.001$). Similarly, in study II, the median C_{max} of M4 was reduced from 49 U/ml (control phase) to 3.5 U/ml (gemfibrozil phase; $P < 0.05$). Also the AUC_{0-72} of M4 and the M4/M6 AUC_{0-72} ratio were clearly reduced in the gemfibrozil phase, compared to the placebo ($P < 0.05$).

M5: In study I, gemfibrozil increased the C_{max} of M5a and M5b by 5.0-fold ($P < 0.001$) and 2.5-fold ($P = 0.001$), respectively, and their AUC_{0-24} by 9.3-fold ($P < 0.001$) and 4.8-fold ($P < 0.001$),

respectively. In study II the similar effect was observed, as the C_{max} and AUC_{0-72} of both M5a and M5b metabolites were increased in the gemfibrozil phase ($P<0.05$).

Figure 1.2.1 Effect of inhibitor drugs on the C_{max} , $t_{1/2}$ and AUC of montelukast (ratios of geometric mean values of C_{max} , $t_{1/2}$ and AUC of montelukast during the inhibitor phases to those during the control (placebo phase))



Gem I gemfibrozil phase in Study I
 Gem II gemfibrozil phase in Study II
 Itra II itraconazole phase in Study II
 Gem+Itra II gemfibrozil and itraconazole phase in Study II

1.3 Effects of itraconazole

Study II included an itraconazole phase, which alone, compared to the placebo, had no significant effect on the pharmacokinetics of parent montelukast (Figure 1.2.1) or the metabolites M6 or M4. However, itraconazole did affect the pharmacokinetic variables of M5a and M5b, compared to the placebo: the median C_{max} and AUC_{0-72} of both M5a and M5b were more than 90% smaller during the itraconazole phase than during the placebo phase ($P<0.05$).

1.4 Effect of gemfibrozil-itraconazole combination

Study II included a gemfibrozil-itraconazole combination phase. This combination, compared to the placebo, had similar effects on the pharmacokinetics of montelukast (Figure 1.2.1) and

metabolites M6 and M4 as gemfibrozil alone. The geometric mean ratio to control of montelukast AUC_∞ was 4.0-fold (95% CI 2.7–6.0, P<0.001) and that of its t_{1/2} was 2.4-fold (95% CI 2.0–3.0, P<0.001). The AUC_∞ and t_{1/2} of montelukast differed from those observed during the itraconazole phase, but not from those observed during the gemfibrozil phase.

The effect of the gemfibrozil-itraconazole combination on the pharmacokinetic variables of the metabolites M5a and M5b was similar to that of itraconazole alone. However, the C_{max} and AUC values of M5a and M5b were reduced to a lesser degree during the gemfibrozil-itraconazole combination phase than during the itraconazole-alone phase, because of the opposite effects of itraconazole and gemfibrozil on the concentrations of these metabolites.

1.5 Effects of genotype

In study I the pharmacokinetic variables of montelukast in *CYP2C8**3, *CYP2C8**4 or *CYP3A5**1 allele carriers were similar to those in non-carriers. Two of the subjects had the *CYP2C8**1/*3 genotype and one had the *CYP2C8**1/*4 genotype. One of the subjects had the *CYP3A5**1/*3 (*CYP3A5* expressor) genotype, whereas the other subjects had the *CYP3A5**3/*3 non-expressor genotype.

1.6 Plasma gemfibrozil, gemfibrozil 1-O-β glucuronide, itraconazole and OH-itraconazole concentrations

The individual concentrations of the inhibitor drugs were consistent with good compliance by all subjects in both studies I and II.

In study I, at all time points during the dosing interval the mean plasma concentrations of gemfibrozil 1-O-β glucuronide were at least 20% higher than those of gemfibrozil. During the gemfibrozil phase, the mean±SD C_{max} of gemfibrozil was 25.0±9.3 mg/l and its AUC₀₋₁₃ 83.7±32.3 mg h/l. The mean C_{max} of gemfibrozil 1-O-β glucuronide was 31.4±8.8 mg/l and its AUC₀₋₁₃ 149.1±39.3 mg h/l.

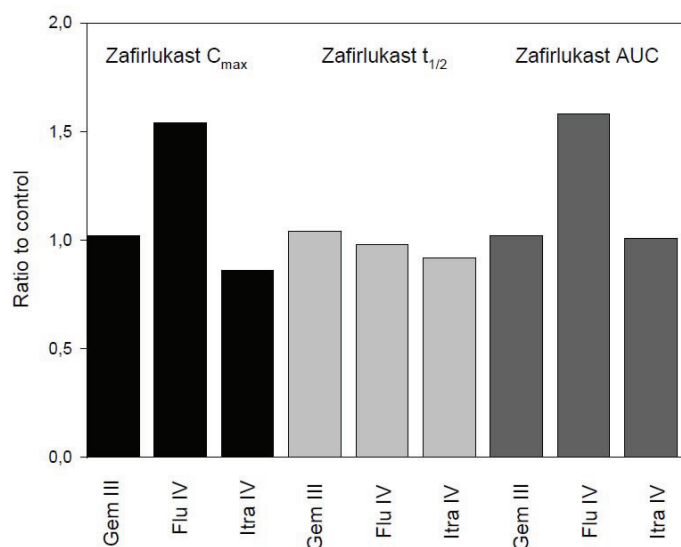
In study II, the concomitant use of itraconazole with gemfibrozil did not affect the plasma concentrations of gemfibrozil and gemfibrozil 1-O-β glucuronide, when compared to the gemfibrozil-alone phase. However, the geometric mean plasma concentrations of itraconazole and OH-itraconazole were more than 50% lower during the gemfibrozil-itraconazole phase than during the itraconazole-alone phase.

2 Pharmacokinetics of zafirlukast (Studies III and IV)

2.1 Effects of gemfibrozil

In study III, gemfibrozil had no significant effect on any of the pharmacokinetic variables of zafirlukast: the mean AUC_{∞} of zafirlukast in the gemfibrozil phase, expressed as the geometric mean ratio, was 102% (range 72–137%, 95% CI 89–116%, $P=0.79$) of the control. The mean C_{max} of zafirlukast was 102% (range 65–134%, 95% CI 87–120%, $P=0.72$), and its mean $t_{1/2}$ was 104% (range 85–131%, 95% CI 91–117%, $P=0.50$) of the control (Figure 2.1.1). The t_{max} of zafirlukast was not changed significantly by gemfibrozil.

Figure 2.1.1 Effect of inhibitor drugs on the C_{max} , $t_{1/2}$ and AUC of zafirlukast (ratios of geometric mean values of C_{max} , $t_{1/2}$ and AUC of zafirlukast during the inhibitor phases to those during the control (placebo phase))



Gem III gemfibrozil phase in Study III
Flu IV fluconazole phase in Study IV
Itra IV itraconazole phase in Study IV

2.2 Effects of fluconazole

In study IV, fluconazole significantly increased the C_{max} and AUC_{∞} of zafirlukast: during the fluconazole phase, the geometric mean ratio to control of the AUC_{∞} of zafirlukast was 1.6 (95% CI 1.3–2.0, $P<0.001$), and that of its C_{max} was 1.5 (95% CI 1.2–2.0, $P<0.05$) (Figure 2.1.1). The C_{max} of zafirlukast was increased in fluconazole phase in all but one subject (range

of fold-increase 0.7–2.4). Fluconazole did not have statistically significant effect on the t_{\max} or $t_{1/2}$ of zafirlukast.

2.3 Effects of itraconazole

In study IV, itraconazole had no significant effect on the pharmacokinetic variables of zafirlukast: the C_{\max} , t_{\max} , $t_{1/2}$ and AUC_{∞} of zafirlukast were similar during the control and the itraconazole phases (Figure 2.1.1). In line with that, the C_{\max} and AUC_{∞} of zafirlukast in the itraconazole phase differed significantly from those in the fluconazole phase.

2.4 Effects of genotype

In study IV, the body weight standardised AUC_{∞} of zafirlukast in the placebo phase did not differ statistically significantly between *CYP2C9**2 and *CYP2C9**3 allele carriers and non-carriers. Neither was there significant difference between the *CYP2C19**2 allele carriers, *CYP2C19**17 allele carriers or non-carriers. There was no clear correlation between the extent of the fluconazole-zafirlukast interaction and the different *CYP2C9* and *CYP2C19* genotypes.

2.5 Plasma gemfibrozil, gemfibrozil 1-O- β glucuronide, fluconazole, itraconazole and OH-itraconazole concentrations

The individual concentrations of the inhibitor drugs were consistent with good compliance by all subjects in both studies III and IV.

In Study III, during the gemfibrozil phase, the mean \pm SD C_{\max} of gemfibrozil was 23.4 \pm 6.9 μ g/ml and its AUC_{0-13} was 98.9 \pm 42.2 μ g h/ml. The mean C_{\max} of gemfibrozil 1-O- β glucuronide was 18.8 \pm 4.1 μ g/ml and its AUC_{0-13} was 111.3 \pm 38.4 μ g h/ml. The mean \pm SD predose morning concentrations of gemfibrozil and gemfibrozil 1-O- β glucuronide were 1.8 \pm 1.7 and 2.1 \pm 1.9 μ g/ml on day 3, 1.6 \pm 0.9 and 2.2 \pm 1.5 μ g/ml on day 4, 1.2 \pm 0.6 and 1.7 \pm 1.0 μ g/ml on day five and 1.5 \pm 0.6 and 2.0 \pm 1.1 μ g/ml on day six, respectively.

In Study IV, the geometric mean C_{\max} of fluconazole was 9.5 mg/l (CV 24.5%), and that of the AUC_{0-25} of fluconazole was 96.9 mg h/l (CV 24.0%). The geometric mean C_{\max} of itraconazole and OH-itraconazole were 337 ng/ml (CV 33.3%) and 710 ng/ml (CV 19.9%), respectively. The geometric mean AUC_{0-13} of itraconazole and OH-itraconazole were 2370 ng h/ml (CV 44.6%) and 6150 ng h/ml (CV 22.9%), respectively.

DISCUSSION

1 Methodological considerations

1.1 Study design

The studies were carried out as clinical drug interaction studies in healthy volunteers. The design was a balanced, randomised, placebo-controlled cross-over study in all studies. Thus all subjects acted as their own control, enabling a more reliable comparison between the different study phases. This was especially justified, as there are significant interindividual variations in the pharmacokinetics of montelukast and zafirlukast. In our studies the interindividual differences in e.g. C_{\max} during the placebo phase were 2.4-fold and 7.2-fold for montelukast, and 1.8-fold and 4.1-fold for zafirlukast at the highest. These are of similar magnitude with those reported earlier (Schoors *et al.* 1995; Cheng *et al.* 1996; Accolate Label 2011; Singulair Label 2012).

The pre-treatment, i.e. the probe inhibitor drug was initiated two days prior to the study day in all studies, and administered one hour prior to the study drug during the study day, in order to ensure adequate inhibitory effect prior to the study treatment. The inhibitor drug was also administered up to the day before the last blood sampling, to ensure that the inhibition was ongoing throughout the sampling period. In study I, however, the duration of the pre-treatment with gemfibrozil was three days, i.e. to the end of the study day. The results were, however, similar to those in study II with a five-day pre-treatment period. As recent publications confirm that the inhibition of CYP2C8 by gemfibrozil occurs rapidly, with sub-therapeutic doses and abates slowly with full recovery only after four days, it can be assumed that the inhibition with the gemfibrozil dose of 600 mg twice daily for three days in study I lasted over the whole sampling period (Backman *et al.* 2009; Honkalammi *et al.* 2011b; Honkalammi *et al.* 2011a; Honkalammi *et al.* 2012a).

The wash-out period was four weeks in all studies in order to minimize possible carry-over effects. It was chosen based on the pharmacokinetics and the inhibitory mechanism of the inhibitor drugs, making possible the elimination of the inhibitor as well as taking into account the time needed for the synthesis of new CYP enzyme in case of irreversible mechanism-based inhibition.

The inhibitor drugs were all recommended probe inhibitors by both the EMA and FDA guidelines (EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012). There is no selective inhibitor for CYP2C9 currently known, and fluconazole inhibits both CYP2C9 and CYP3A4. However, the CYP3A4 inhibitor itraconazole used in study IV enabled the estimates of the possible proportion CYP3A4 inhibition in the pharmacokinetics of zafirlukast.

1.2 Statistical analysis

The statistical analyses were carried out according to the recommended methods of the peer-reviewed journals at the time of the preparation of each manuscript. The evolution of these recommendations during the course of these studies is reflected by the variable approaches regarding the method chosen for the outcome metrics in each study.

In the case of montelukast, the results for the pharmacokinetic variables were expressed in study I as the mean \pm SD, except for t_{\max} , which was expressed as the median (range). In study II, all variables for montelukast, except t_{\max} , were transformed with natural logarithm, and the results were expressed as a geometric mean and CV in the text, and as a mean \pm SEM in figures. The comparisons between the two treatment phases were performed by the t-test in study I. In study II the results of the four phases were compared with a repeated measures ANOVA followed by a posteriori testing with the paired t-test with the Bonferroni correction. As the C_{\max} , $t_{1/2}$ and AUC are asymmetrically distributed outcome metrics, the approach utilised in study II would be the preferable choice. The geometric mean for the C_{\max} , $t_{1/2}$ and AUC of montelukast in study I were calculated for Figure 1.2.1, and were in line with those observed in study II.

Regarding zafirlukast, in study III the results for the pharmacokinetic variables were expressed as the mean \pm SD, except for t_{\max} , which was expressed as the median (range). In study IV all variables for zafirlukast, except t_{\max} , were transformed with natural logarithm, and the results were expressed as geometric mean and CV or 90% CI (in figures). The t_{\max} was expressed as median (range). The pharmacokinetics of zafirlukast alone in studies III and IV were comparable: the C_{\max} , $t_{1/2}$ and AUC values in the placebo phases were in accordance with each other in these two studies. Also for zafirlukast, the geometric mean for the C_{\max} , $t_{1/2}$ and AUC of zafirlukast in study III were calculated for Figure 2.1.1.

2 Interpretation of the results

2.1 Mechanism of the interactions

2.1.1 Montelukast

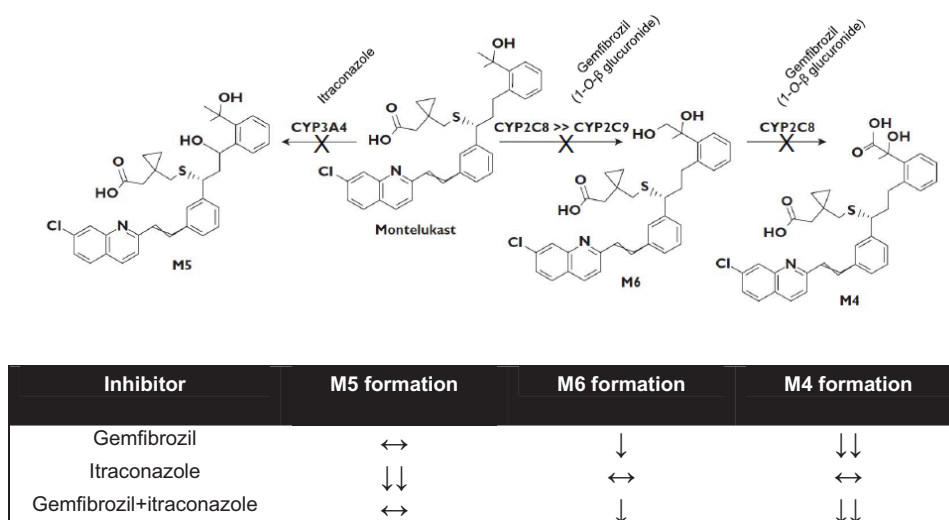
In studies I and II, the effect of gemfibrozil on the pharmacokinetics of montelukast was significant with regard to the AUC (over fourfold increase), $t_{1/2}$ (over twofold prolongation) and C_{\max} (about 1.5-fold increase), but the t_{\max} did not differ statistically in either study. The formation of the primary metabolite M6 was clearly delayed by gemfibrozil in both studies, the t_{\max} of M6 prolonging from about four hours to twelve hours. The formation of the main metabolite M4 was reduced over 90% in both studies. The only effect of itraconazole was the decreased C_{\max} and AUC of the minor metabolite M5 in study II. These findings were complemented by the *in vitro* studies, where gemfibrozil and gemfibrozil 1-O- β glucuronide

inhibited the formation of M6 and its further metabolism to M4, but had no effect on the formation of M5.

In addition to the mechanism-based inhibition of CYP2C8, gemfibrozil has inhibited also the OATP2B1 mediated transport of rosuvastatin *in vitro* (Ho *et al.* 2006). Had the intestinal intake of montelukast been inhibited by gemfibrozil, it would have resulted in decreased plasma concentrations of montelukast. If the inhibition of the hepatic uptake in turn had been inhibited by gemfibrozil, it could have resulted in decreased hepatic clearance of montelukast, and could thus have contributed to the observed interaction. However, the effect of gemfibrozil on the possible OATP2B1-mediated hepatic uptake of montelukast can be assumed to be weak and short in duration in humans, based on the short $t_{1/2}$ of gemfibrozil and only a small inhibitory effect of gemfibrozil on the hepatic uptake observed for fluvastatin *in vitro* (Noe *et al.* 2007) and that its inhibitory effect on OATP2B1 was only as strong as on CYP2C9 *in vitro* (Wen *et al.* 2001; Mougey *et al.* 2009). Additionally, it has been argued, however, that montelukast pharmacokinetics have not been affected by changes in activity of OATP2B1 (Chu *et al.* 2012; Tapaninen *et al.* 2012).

Altogether these results strongly indicate that the interaction between montelukast and gemfibrozil occurs at the level of metabolism in the liver. Gemfibrozil is a strong and selective mechanism-based inhibitor of CYP2C8, and as inhibition of CYP3A4 did not significantly affect the total elimination of montelukast, the interaction resulted from the inhibition of metabolism of montelukast to M6, and further metabolism of M6 to M4 by CYP2C8. Together with these findings, and those resulting from *in vitro* studies (Filppula *et al.* 2011), it can be estimated that CYP2C8 accounts for about 80% of the metabolism of montelukast in humans. Based on these *in vivo* findings combined with the *in vitro* findings published earlier (Filppula *et al.* 2011), the metabolism of montelukast and the enzymes participating in it can be presented as in Figure and table 2.1.1.1.

Figure and table 2.1.1.1. The metabolism of montelukast to M6, M4 and M5, the enzymes participating in their formation in humans, and the effect of inhibitor drugs on the formation of the metabolites



2.1.2 Zafirlukast

In study III gemfibrozil had no effect on the pharmacokinetics of zafirlukast: the 95% CIs of the geometric mean ratios of the zafirlukast C_{\max} , $t_{1/2}$ and AUC compared to the placebo were within those required for determining bioequivalence after a single dose (CHMP Guideline on the investigation of bioequivalence 2010). Thus it could be concluded that CYP2C8 does not play a significant role in the elimination of zafirlukast in humans.

In study IV fluconazole increased the C_{\max} of zafirlukast 1.5-fold and the AUC 1.6-fold, but affected neither the $t_{1/2}$ nor t_{\max} of zafirlukast. Itraconazole, compared to the placebo had no effect on any of the pharmacokinetic variables of zafirlukast.

Based on these two studies it can be concluded that only CYP2C9 plays a role in the biotransformation of zafirlukast, whereas inhibition of CYP2C8 and CYP3A4 had no effect on its pharmacokinetics. The effect on fluconazole can be assumed to be mediated via CYP2C9, although it does also inhibit CYP2C19. However, in study IV the subjects were equally distributed between CYP2C19 wild-type, loss-of-function and gain-of-function genotypes, and thus, if CYP2C19 had a significant role in the metabolism of zafirlukast, a trend could have been expected to be seen between these genotype groups.

The conclusion from these results is that the mechanism of inhibition by fluconazole was thus inhibition of CYP2C9-mediated metabolism of zafirlukast, resulting in 50% increase in its AUC. Inhibition of CYP3A4 did not affect the pharmacokinetics of zafirlukast, and different CYP2C19 genotypes did not reveal any trend towards different pharmacokinetics in differently functioning genotypes.

2.2 The results in light of the previous knowledge

2.2.1 Montelukast

Around the time of the marketing authorisation of montelukast in 1997 the knowledge on the enzymes involved in its metabolism was based on *in vitro* studies with high, over 100-fold the total plasma concentration of montelukast and over 20000-fold its unbound fraction. Further, CYP2C8 was not included in the assays. The main metabolites in human had been identified: M4 as the major metabolite, resulting from further oxidation of a primary metabolite, M6. M5 was identified as one of the four minor metabolites. *In vitro* the formation of M5 was attributed to CYP3A4 and formation of M6 to CYP2C9 (Balani *et al.* 1997; Chiba *et al.* 1997; Singulair Label 2012).

During the last decade the importance of CYP2C8 in drug metabolism became increasingly acknowledged. The estimated percentage of CYP2C8 in drug oxidations (8%, Figure 7.1) dates from 2005 and earlier studies, and may possibly be an underestimation. When the inhibitory effect of 209 drugs on CYP2C8 was screened *in vitro*, montelukast was the most potent CYP2C8 inhibitor (Walsky *et al.* 2005a; Walsky *et al.* 2005b). The inhibition was competitive, and the inhibitory effect of montelukast was highly dependent on microsomal protein concentration, possibly due to nonspecific microsomal binding (Walsky *et al.* 2005b). In *in vivo* studies montelukast did not show any inhibitory effect on the pharmacokinetics of several

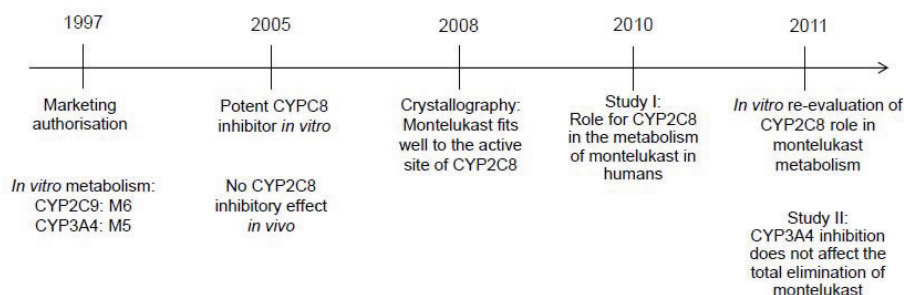
CYP2C8 substrates, (Jaakkola *et al.* 2006a; Kajosaari *et al.* 2006; Kim *et al.* 2007), most probably explained by the low unbound concentration <5 nmol/l, which is lower than its K_i value for CYP2C8, 0.009–0.15 $\mu\text{mol/l}$ (Walsky *et al.* 2005b).

The molecular structure of CYP2C8 determined by x-ray crystallography was published in 2004. A large active site was observed, as could be expected from the size of its known substrates, e.g. paclitaxel and cerivastatin (Schoch *et al.* 2004). The substrate binding of CYP2C8 was further determined with crystallography, and montelukast with its tripartite structure was observed to fit well into the active site of CYP2C8 without major changes in the tertiary structure of the enzyme. The orientation of montelukast into the active site was determined by the size of the largest branch and polarity of the two smaller branches, resulting in positioning the benzyl ring of montelukast close to the heme iron of the CYP2C8 active site (Schoch *et al.* 2008). This at the latest, in relation to the earlier above, suggested that montelukast could be a substrate of CYP2C8.

Simultaneously with studies I and II the *in vitro* metabolism of montelukast was investigated. The studies were conducted with lower, clinically more relevant montelukast concentrations than those conducted over 10 years earlier. The effects of CYP inhibitors on montelukast metabolism were investigated in HLMs, and montelukast oxidations in rhCYP enzymes. CYP2C8 inhibition inhibited the depletion of montelukast and formation of M6 more potently than CYP2C9 inhibition. RhCYP2C8 catalysed the depletion of montelukast and formation of M6 with sixfold intrinsic clearance compared to CYP2C9, and no other isoform produced M6. Based on these *in vitro* findings it was estimated that CYP2C8 would account for 72% of the oxidative metabolism of montelukast *in vivo* (Filppula *et al.* 2011). When the contribution of CYP2C8 in the metabolism of montelukast *in vitro* was further evaluated in another study, the formation of M6 was predominantly mediated by CYP2C8, not CYP2C9 or CYP3A4. In addition, gemfibrozil did not reduce the formation of M5, which indicates that M5 formation is mediated by CYP3A4 (VandenBrink *et al.* 2011).

The results of studies I and II, performed and published simultaneously with the *in vitro* studies described above, complement the understanding of the metabolism of montelukast in humans. The formation of the primary metabolite M6 and its further oxidation to the main metabolite M4 is catalysed by CYP2C8, which accounts for about 80% of montelukast metabolism. CYP3A4 only catalyses the formation of a minor metabolite M5, and inhibition of CYP3A4 does not alter the total elimination of montelukast. These findings were in contrast to the previous knowledge on the metabolism of montelukast included in its prescribing information, i.e. metabolism via CYP2C9 and CYP3A4. The evolution of the understanding of the montelukast metabolism in humans is summarised in Figure 2.2.1.1.

Figure 2.2.1.1 Evolution of the knowledge of montelukast metabolism in humans (Chiba *et al.* 1997; Walsky *et al.* 2005a; Walsky *et al.* 2005b; Kajosaari *et al.* 2006; Schoch *et al.* 2008; Filppula *et al.* 2011; Study I and Study II)



2.2.2 Montelukast as a CYP2C8 probe substrate

The guidelines for drug interaction studies from both the EMA and the FDA include characterisations for an *in vivo* probe substrate. The probe substrate should be exclusively or almost exclusively eliminated either through metabolism catalysed by one specific enzyme, or excretion by one specific transporter. In case a second enzyme or transporter participates in the elimination of the drug, its contribution to the total clearance should be very small. The elimination of the probe drug should be well characterised *in vivo*, and it should display linear pharmacokinetics. Therefore, the pharmacokinetics of the probe substrate are markedly altered by the coadministration of known specific inhibitors. (EMA Guideline on the investigation of Drug Interactions (final) 2012; FDA Draft Guidance for Industry 2012)

Both these guidelines also recommend CYP2C8 probe substrates for *in vivo* studies: repaglinide is mentioned by the FDA draft guidance, stating that it is also a substrate of OATP1B1, and in addition paclitaxel is mentioned as a CYP2C8 substrate with a narrow therapeutic range (FDA Draft Guidance for Industry 2012). EMA guideline lists amodiaquine and repaglinide as possible CYP2C8 probe drugs, although disclaiming that there is no well-documented probe drug at present, and that other probe drugs may be used if justified by scientific literature (EMA Guideline on the investigation of Drug Interactions (final) 2012). Amodiaquine is an antimalarial drug available in Africa, but not in the market in the EU or the US. Thus repaglinide remains the most easily available *in vivo* probe for CYP2C8.

Repaglinide has been granted marketing authorisation in 1997 in the US and in 1998 in the EU. As described earlier, it is metabolised primarily by CYP2C8 in the liver, and also by CYP3A4 during the first pass metabolism. Gemfibrozil has increased its AUC over eightfold, itraconazole 1.4-fold, and these CYP2C8 and CYP3A4 inhibitors together 19-fold (Hatorp *et al.* 2003; Niemi *et al.* 2003b; Tornio *et al.* 2008a). As an OATP1B1 substrate its pharmacokinetics are influenced by *SLCO1B1* polymorphism: e.g. a common SNP c.521T>C results in 107% higher AUC in subjects with the *SLCO1B1* c.521CC genotype (Niemi *et al.* 2005). The beneficial feature of repaglinide is its short $t_{1/2}$ of 1–1.5 h, which allows a shorter sampling period to cover

the full plasma concentration-time course. On the disadvantageous side, again, is its mechanism of action. It stimulates the release of insulin from the pancreas, aiming in blood glucose-lowering effect throughout the meal period in type II diabetes patients. However, this effect may result in hypoglycemia during drug interaction studies, which complicates the study conduct by requiring frequent blood glucose level monitoring and possible rescue interventions. Due to this risk of hypoglycemia the concomitant use of repaglinide with gemfibrozil is actually contraindicated (Lopid Label 2010).

Reflecting the above characteristics for a probe substrate, montelukast promisingly fulfils several. It displays linear pharmacokinetics, does not undergo first-pass metabolism, and its elimination *in vivo* has been characterised quite extensively. Its pharmacokinetics were affected markedly by CYP2C8 inhibition, resulting in over fourfold increase of its AUC. Involvement of CYP3A4 in its elimination was shown to be minimal. However, the possible role of transporters in the pharmacokinetics of montelukast remains to be further investigated. Moreover, montelukast has a 3–5-fold longer half-life than repaglinide, thus requiring longer sampling period to cover the full plasma concentration-time course also during inhibition of its metabolism. This is, however, counterbalanced by its relative safety and wide safety margin requiring no additional safety monitoring during drug interaction studies.

2.2.3 Zafirlukast

In vivo the metabolism and excretion of zafirlukast has been studied in dogs, rats and mice (Savidge *et al.* 1998). In all the species studied, biliary clearance of unchanged zafirlukast and its metabolites was the major route of elimination. The primary routes of metabolism included hydrolysis of the amide linkage at the 5-aminoindole position and hydroxylation at one or more sites. The most abundantly present metabolite in dog bile was M6, formed by hydroxylation of the cyclopentyl ring. Parent zafirlukast, however, was the most abundant compound in bile, and totally about 35% of the zafirlukast-derived radioactivity was excreted in the faeces. (Savidge *et al.* 1998)

At the time of the marketing authorisation of zafirlukast in 1996 the knowledge on its metabolism was based mainly on unpublished *in vitro* studies. It was acknowledged as extensively metabolised, mostly to hydroxylated metabolites excreted in the faeces. In HLMs the formation of the hydroxylated metabolites was shown to be catalysed by CYP2C9, with participation of also CYP3A4 and CYP2C19 in its biotransformation (Accolate Pharmacology Review(s) 1998; Kassahun *et al.* 2005; Accolate Label 2011). With regard to the *in vivo* metabolism of zafirlukast, there are no published studies investigating the role of CYP isoforms in the metabolism of zafirlukast in humans.

Zafirlukast has inhibited CYP2C9 and CYP3A4 *in vitro*, but with higher IC₅₀ than the concentrations observed clinically (Shader *et al.* 1999). Like montelukast, zafirlukast was among the most potent inhibitors of CYP2C8 (Walsky *et al.* 2005a). Thereafter montelukast was shown to fit well into the active site of CYP2C8 (Schoch *et al.* 2008), leading to the finding that CYP2C8 has a major role in the metabolism of montelukast (Studies I and II). These findings stimulated the study of the role of CYP2C8 also in the metabolism of zafirlukast, which was shown to be non-existing (Study III). Thereafter the role of CYP2C9 and CYP3A4 in the pharmacokinetics of zafirlukast in humans was studied. Only inhibition of CYP2C9 was shown to affect the pharmacokinetics of zafirlukast *in vivo*, and with this finding the main metabolic pathway of zafirlukast in humans was characterised (Study IV). The prescribing information of

zafirlukast providing CYP2C9, based on *in vitro* studies, as the main enzyme participating in its metabolism could now be updated with the above knowledge of the role of CYP2C9 in the *in vivo* metabolism of zafirlukast in humans.

With regard to the potential of zafirlukast as a CYP2C9 probe drug, it does display linear pharmacokinetics. However, its metabolism is not sufficiently characterised. Its elimination in humans has now been studied with regard to three CYP enzymes, but the role of e.g. CYP2C19 remains unknown. In drug interaction studies with CYP2C8, CYP2C9 and CYP3A4 only the inhibition of CYP2C9 resulted in 1.6-fold increase of the AUC of zafirlukast (Study IV). Thus it does not express the desired sensitivity of a probe drug, i.e. usually an AUC increase fivefold or more with a known CYP inhibitor (FDA Draft Guidance for Industry 2012). When almost 90% of zafirlukast is eliminated by metabolism and excretion in the bile, and 10% in the urine as metabolites (Accolate Clinical Pharmacology and Biopharmaceutics Review(s) 1999), and, according to another reference 33% is metabolised in human (Accolate Pharmacology Review(s) 1998), it cannot be concluded that zafirlukast would be exclusively or almost exclusively eliminated either through metabolism catalysed by one specific enzyme, or excretion by one specific transporter, as an ideal probe drug should. Thus, the current knowledge does not support zafirlukast as a promising CYP2C9 probe substrate.

2.3 Generalisation of the results and clinical implications

As these studies were carried out in healthy volunteers, their generalisation to the asthma patient population can be argued. However, there are no indications of differences in the pharmacokinetics of montelukast and zafirlukast in healthy volunteers and asthma patients, and similar interindividual differences as reported earlier were observed for both montelukast and zafirlukast also in our studies (Accolate Pharmacology Review(s) 1998; Calhoun 1998; Singulair Pharmacology Review(s) 1998; Accolate Label 2011; Singulair Label 2012). As the inhibition of both the study drugs occurs most probably at the level of elimination in the liver, the results could be altered in pathologic conditions deteriorating the liver function. The reversible inhibition is diminished much more drastically than mechanism-based inhibition in patients with hepatic diseases (Palatini *et al.* 2010).

In studies I and II the AUC of montelukast was increased 4.3–4.5-fold by the strong CYP2C8 inhibitor gemfibrozil. The relevance of the magnitude of the interaction could be reflected to the dose-dependency of the efficacy and safety observed in clinical studies of montelukast. No additional benefit was observed from montelukast doses above 10 mg daily in clinical studies in adults. Additionally, montelukast has been administered at doses up to 200 mg/day to adult patients in chronic asthma studies (22 weeks), and even up to 900 mg/day in short-term studies (approx. a week), without observing clinically important adverse experiences (Singulair Label 2012). Both montelukast and zafirlukast have wide safety margin, thus potentially diminishing the clinical relevance of these findings.

For montelukast it could be concluded that the although the interaction with the CYP2C8 inhibitor gemfibrozil resulted in significant increase of the total exposure of montelukast, it is not likely to be clinically significant in most of the patients due to the wide safety margin observed for montelukast in clinical studies. However, increased risk for concentration dependent ADRs cannot be ruled out, and dose reduction of 50–80% could be considered for montelukast if used concomitantly with a CYP2C8 inhibitor, especially in patients with impaired hepatic function.

The need for this dose adjustment can be weighed against the expected duration of the inhibition, depending on e.g. the planned duration of the treatment with the CYP2C8 inhibitor drug and its inhibitory mechanism.

Fluconazole increased the AUC of zafirlukast by 1.6-fold. It is known from clinical studies that the pharmacodynamics of zafirlukast show dose response to a certain degree, but e.g. twofold increase in the dose (from 20 mg to 40 mg twice daily or from 40 to 80 mg twice daily) does not affect the asthma symptoms or decrease the need for β_2 -agonist use. (Calhoun 1998). Also the safety of zafirlukast has been similar in clinical studies with the dose varying from 20 mg to 80 mg twice daily, with asymptomatic elevations of liver enzymes in patients receiving the highest dose, 80 mg twice daily (Dekhuijzen *et al.* 2002). Although the inhibition of CYP3A4 did not affect the pharmacokinetics of zafirlukast in humans, CYP3A4 has catalysed the formation of minor metabolites of zafirlukast *in vitro*, including a potentially hepatotoxic unsaturated iminium agent (Kassahun *et al.* 2005). If the major metabolic pathway CYP2C9 is inhibited by a concomitant drug, this minor pathway might gain more importance and result in increased amount of this potentially hepatotoxic agent.

In conclusion, the interaction observed between zafirlukast and fluconazole, or other CYP2C9 inhibitor, is not likely to have significant clinical relevance with regard to safety or efficacy, but as for montelukast, an increased risk for concentration dependent ADRs cannot be ruled out. However, during the clinical experience of both montelukast and zafirlukast, extending over 15 years, no such safety concern has been observed so far.

Both montelukast and zafirlukast were developed and granted marketing authorisations before the regulatory guidelines regarding drug interaction studies during drug development were published. Since the introduction of these drugs to the market, the importance of CYP enzymes in drug interactions has been increasingly recognised. Had these drugs been developed during the existence of the current regulatory guidelines, CYP2C8 had been included in the early *in vitro* screening studies, and the strong inhibitory effect on CYP2C8 observed for both these drugs may have led to *in vivo* drug interaction studies in humans to further evaluate its effect. The current knowledge of CYP2C8 as the main route of elimination of montelukast, and the importance of CYP2C9 in the metabolism of zafirlukast in humans would thus have been characterised and included in the prescribing information at the time of the marketing authorisations of these drugs.

CONCLUSIONS

The following conclusions can be made based on the studies in this thesis:

1. Inhibition of CYP2C8 by gemfibrozil results in over fourfold increase of montelukast AUC, and the formation of the major metabolite M4 almost ceases. In contrast to the previous knowledge, the main route of elimination of montelukast in humans is biotransformation catalysed by CYP2C8.
2. CYP3A4 mediates the formation of a minor montelukast metabolite M5, but the inhibition of CYP3A4 does not have a significant effect on the total elimination of montelukast. Thus CYP3A4 is not important in the metabolism of montelukast.
3. Montelukast could serve as a safe and sensitive CYP2C8 probe drug for drug interaction studies during drug development.
4. The inhibition of CYP2C9 results in 1.6-fold increase of zafirlukast AUC, whereas CYP2C8 and CYP3A4 do not participate in its biotransformation. The results thus revealed that CYP2C9 is important in the metabolism of zafirlukast in humans.
5. These results highlight the relevance of drug interaction studies and the regulatory guidelines related to them. Especially drugs that have been developed before the existence of these guidelines may be deficiently characterised with regard to their metabolism, leaving the possibility of unrecognised CYP-mediated interactions.

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